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THE UNIVERSITY OF ALBERTA

IONIC CONDUCTANCES IN DEVELOPING SKELETAL MUSCLE

by

© CAROL MACLEAN THOMSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled IONIC CONDUCTANCES IN DEVELOPING SKELETAL MUSCLE, submitted by Carol M. Thomson in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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TO MY FAMILY

GEORGE, KATHRYN and PETER

I took the one less travelled by

And that has made all the difference.

Robert Frost

ABSTRACT

The development of resting membrane conductance to the major ions has been studied in cultures of chick pectoral muscle. The contribution of each ion to total membrane conductance was estimated by removal of the ion under investigation and replacement by an impermeant substitute. Known antagonists of resting ion conductances were used in an attempt to confirm the results of the replacement experiments. The slow regenerative response and the appearance of the transient sodium channel were also investigated.

1-β-D-arabinofuranosyl cytosine (ara-C) has previously been used to suppress the mononuclear cell population in primary muscle cultures. A preliminary study indicated that this agent could prevent the appearance of the fully mature transient sodium channel. Therefore the use of ara-C was discontinued thereafter.

Membrane conductance was initially high in immature fibres (*ca.* 1.25 $mScm^{-2}$) but fell during maturation to a final level of about 0.2 $mScm^{-2}$. Resting membrane potential rose and membrane conductance fell most rapidly between days 3 and 5 of culture.

The relative contribution to membrane conductance by potassium remained constant at about 30%. Chloride conductance rose from 50% to 65-70% and the contribution from sodium fell from 20-30% to undetectable levels by the sixth day of culture. No detectable conductance to calcium was found in the resting membrane.

Agents reputed to block specifically chloride and potassium channels (anthracene-9-carboxylic acid, zinc, aminopyridines) were not specific in immature fibres. All increased membrane conductance to sodium

ions. Zinc was cytotoxic to immature fibres and the use of this agent was stopped. By day 6 and onwards, anthracene-9-carboxylic acid and the aminopyridines reduced membrane conductance in a manner compatible with blockade of their respective channels. Verapamil, reputed to block slow current channels activated by depolarization in excitable membranes, was found to block resting sodium conductance in immature myotubes.

Condylactis toxin (CTX) caused depolarization and a reduction in input resistance in fibres from day 3 onwards. Both effects increased as the fibres matured. CTX increased the rate of rise of the slow regenerative activity in immature myotubes and induced action potential generation in older fibres. Tetrodotoxin and veratridine sensitivity appeared on the sixth day of culture. Prior to that regenerative activity was blocked by manganese, cobalt or lanthanum. In immature myotubes, there is a precursor regenerative channel, sensitive to CTX, which allows the passage of both sodium and calcium ions on activation. This channel is either replaced subsequently by the transient sodium channel as a consequence of turnover of membrane proteins, or the precursor channel is modified *in situ* to become the transient sodium channel.

It was originally postulated that membrane potential rose because the membrane became relatively more permeable to potassium. However the potassium conductance does not rise as suggested previously. The increase in membrane ion selectivity is attributed rather to a loss of sodium conductance. Since the final component conductances more closely resembled those of innervated muscle it is suggested that the passive membrane properties of aneurally cultured muscle can be modified further by trophic influences from nerve.

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I. INTRODUCTION

A. Voltage Sensitive Ionic Channels in Adult Skeletal Muscle

1. Permanent Channels.

i. Potassium Channels. Bernstein (1902, 1912) attributed the fact that an appreciable potential difference was maintained across excitable membranes to a selective permeability of the cell membrane to ions, and that during activity the membrane permeability was increased. He found, using the Nernst equation, that the resting membrane potential of frog muscle was well approximated if the membrane were selectively permeable to potassium ions. However Boyle and Conway (1941) recognized that skeletal muscle was permeable to chloride as well as potassium ions. At equilibrium they found that the concentration ratios and membrane potential conformed to the following Donnan relation.

$$\frac{[K]_o}{[K]_i} = \frac{[Cl]_i}{[Cl]_o} = \exp\left(\frac{VF}{RT}\right)$$

$[\ ]_o$ = concentration of ion outside

$[\ ]_i$ = concentration of ion inside

V = membrane potential

F,R = Faraday, and gas constants

T = temperature °K

They concluded that the ionic diameter was critical for diffusion through the membrane and inclined to the view that the same molecular pore existed for both small cations and anions, and that the pore was probably not charged. However it was found that on displacement of the membrane potential the potassium and chloride conductance behaved differently (Katz, 1949; Hodgkin and Horowicz, 1959b; Hutter and Noble, 1960). Evidence was also available which suggested that the chloride pathway was situated closer to the outer membrane than the potassium one (Hodgkin and Horowicz,

1960; Adrian and Freygang, 1962a, 1962b). The theory of Boyle and Conway (1941) was finally abandoned when it was discovered that replacement of one ion with impermeant substitutes did not affect the fluxes of the other ion (Edwards *et al.*, 1957).

Frog muscle was found to behave as a potassium electrode i.e. membrane potential varied linearly with the logarithm of the external potassium concentration when this was 10 mM or higher (Adrian, 1956; Hodgkin and Horowicz, 1959b). Below this concentration, the relationship deviated markedly from linearity and the difference was attributed to a permeability of the membrane to sodium ions of the order of one hundredth that to potassium.

Mammalian muscle membrane potentials were not well described by the Nernst equation, (Boyd and Martin, 1959). With the recognition of a limited permeability to sodium ions, and the acceptance of the constant field assumption (Goldman, 1943) this led to the establishment of the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949) as the mathematical description of resting membrane potential:

$$E_m = \frac{RT}{F} \ln \frac{P_K[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i}{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o}$$

Tracer flux experiments (Keynes, 1954; Hodgkin and Horowicz, 1959a) and the studies of Adrian (1956, 1960) confirmed the theoretical predictions to a large extent. However in muscle it was found that the inward movement of potassium was more rapid than that for outward movement (Katz, 1949; Hodgkin and Horowicz, 1959b; Adrian, 1960). This fall in conductance when membrane current flows out of the membrane and the rise in conductance when current flows in the opposite direction was called

anomalous rectification since the conductance changes were in the opposite direction to those predicted by the constant field equation (Adrian and Freygang, 1962a). Adrian and Freygang (1962a, 1962b) postulated that the reason for this anomalous rectification was the existence of two channels for potassium which rectified in opposite directions. They proposed that the potassium permeability of the surface membrane was low at rest and rose during an action potential. The majority of the resting potassium permeability was proposed to reside in the T-tubular membrane. It was tacitly assumed that this flow of ions occurred either through pores or channels in the appropriate membrane or via a carrier mechanism which required no metabolic energy. Later work by Armstrong and colleagues (Armstrong and Binstock, 1965; Armstrong, 1966, 1969, 1971; Armstrong and Hille, 1972) using tetraethylammonium (TEA) confirmed that a channel was the route of permeation. The relationship between the steady-state potassium channel and the increase in potassium conductance which accompanies an action potential has not been studied. It is not clear whether the steady state channel undergoes a conformational change during an action potential or whether there are two separate channels for potassium ions.

From experiments in which extracellular chloride was replaced with impermeant anions and assuming that the contribution from sodium was negligible, estimates of the resting potassium conductance ($\bar{g}K$) of muscle have been calculated. Hodgkin and Horowicz (1959b) estimated $\bar{g}K$ as 0.1 $mScm^{-2}$ from external and internal ion concentrations and membrane potential measurements. Later workers measured input resistances, fibre diameters and or space constants and calculated membrane conductance from

cable theory considerations. Mashima and Washio (1964), Stanfield (1970) and Fink and Lüttgau (1976) also found $\bar{g}K$ in frogs to be about 0.1 $mScm^{-2}$ and $\bar{g}K$ accounted for 33% of the total membrane conductance. In mammalian muscle, $\bar{g}K$ was more variable (0.1 to 0.3 $mScm^{-2}$) and it was found to contribute only 8 to 18% of the total membrane conductance (Bryant and Morales-Aguilera, 1971; Bryant and Camerino, 1976; Camerino and Bryant, 1976; Lorković and Tomanek, 1977; Palade and Barchi, 1977a). One report only has been published on avian muscle. Morgan *et al.* (1975) found that $\bar{g}K$ of pigeon biventer cervicis muscle was 0.6 $mScm^{-2}$ and contributed 17% of the total resting membrane conductance.

When the effect on potassium currents of derivatives of TEA with a bulky, hydrophobic sidechain was measured, the most potent derivative was nonyltriethylammonium (C9) (Armstrong, 1966, 1969, 1971, 1974). The ethyl groups around the charged nitrogen gave this portion of the molecule a diameter of 8 Å, approximately the size of a hydrated potassium ion. When the ethyl groups were replaced by methyl groups, the resulting ion was ineffective as a blocker of potassium channels. The hydrophobic chain of C9 was also found to be important in its blocking action, suggesting that it bound to a hydrophobic group near the channel mouth. Inhibition only occurred after the K^+ permeability had been increased by a depolarization which suggested that C9 was swept into the channel along with the K^+ ions.

Recovery from C9 inactivation was accelerated by an increase in the potassium concentration of the external solution or by hyperpolarization. Armstrong (1966, 1969, 1971, 1974) proposed that this was because the C9 was in an energy well due to hydrophobic bonding. The presence of a K^+

ion in the channel entering from the outside would raise the potential energy of the C9 ion by electrostatic repulsion and this would in effect push the C9 ion from the channel. The rate of clearance of the block would therefore depend on the probability of finding a K^+ ion in the channel. This in turn is proportional to $[K]_o$ and increases as the membrane is hyperpolarized. Because strong hyperpolarizations delayed a late phase of the clearing of C9 from the channel, Armstrong suggested that this was because the channel could close with a C9 molecule in place therefore delaying the clearance of these molecules from the channel. This state was not favoured energetically, and the majority of the channels would remain open although inactivated. He then proposed that the potassium channel had a wider portion on the inside of the cell membrane of 8-9 Å diameter into which C9 and hydrated potassium ions could fit. C9 could not be dehydrated and could not penetrate the narrower portion of the channel.

These effects could not easily be explained by a carrier model and provided strong evidence that the K^+ ions pass through the membrane by means of channels (Armstrong, 1971, 1974). The data from the C9 experiments were incompatible with a carrier which transported ions one at a time, and carriers which transport two or more ions are unlikely for electrostatic reasons (Parsegian, 1969). Ion movements occur in milliseconds and the net ion flux estimated per estimated permeability unit or structure (Hodgkin and Horowicz, 1959a) was higher than that which occurred with known carriers eg. nonactin (Armstrong, 1971; Laüger, 1972). Parsegian (1969) calculated that there was an energy barrier of at least 16 Kcal/mole for the movement of an ion through a lipid membrane in a

carrier of reasonable dimensions (5 Å radius) which corresponds to a Q_{10} of 2.4. Frankenhaeuser and Moore (1963) found that the Q_{10} of ion transport through nerve membrane was 1.2 which corresponds to an energy barrier of 3 Kcal/mole. This value was close to that calculated for a pore or ion channel (Parsegian, 1969).

When the selectivity of the potassium channel was studied it was found that other ions interfered with the flow of K^+ ions through this channel (Adelman and Senft, 1968; Bezanilla and Armstrong, 1972). Sodium, lithium, TEA and its derivatives interfered if present inside the squid axon and caesium interfered from both sides. This was explained in a similar manner to that for C9. The interfering cation could enter the wide portion of the pore which was postulated to be less selective than the narrow portion. The cation however could not pass the narrow portion and would prevent the flow of K^+ ions out until a K^+ ion entering from the outside forces the interfering cation into the axoplasm by electrostatic repulsion.

Based on permeability ratios to various metallic and organic cations estimated from the constant field equation, the potassium channel has been estimated to have at least one selectivity portion approximately 3 Å in diameter which is capable of accepting dehydrated potassium ions (Tasaki *et al.*, 1961; Tasaki and Spyropoulos, 1961; Bezanilla and Armstrong, 1972; Hagiwara *et al.*, 1972; Hille, 1973).

The diameter of the mouth of the pore was deduced to be at least 8 Å in diameter (Armstrong, 1971, 1974) and the narrow part of the pore was permeable to ions with a crystal diameter of 2.66 Å (K^+) to 2.96 Å (Rb^+, NH_4^+) but not to ions which were smaller (Na^+, 1.9 Å; Li^+, 1.36 Å) as

well as larger ones (Cs^+, 3.3 Å; TEA, 8 Å) (Bezanilla and Armstrong, 1972; Hille, 1973). Bezanilla and Armstrong (1972) proposed that the narrow part of the pore was about 3 Å in diameter and that as an ion passes through it lost most of its water of hydration. This process would only be favoured if the partially dehydrated ion interacted with the walls of the pore by hydrogen bonding to oxygen atoms which were postulated to line the channel. Ions larger than 3 Å would therefore be hindered sterically. Ions smaller than 2.6 Å would be excluded because they could not interact favourably with the oxygens lining the pore walls, and thus the entry of such ions would not be favoured energetically.

Recently, Hille and Schwarz (1978) have postulated that both the potassium channel involved in the action potential - the delayed rectifier - and the channel which conducts inward current better than outward current - the anomalous rectifier - (Katz, 1949; Adrian and Freygang, 1962a) are multi-ion pores, which transport ions in single file (Hodgkin and Keynes, 1955). Properties which were impossible to explain in terms of one-ion pore or one-ion carrier models included unidirectional flux-ratio exponents > 1.0, and effective valence of blocking reactions > 1.0, properties dependent on E_m-E_K rather than on E_m alone and permeability ratios depending on the ionic composition of the bathing solutions. Hille and Schwarz (1978) suggested that potassium channels contained three internal sites or energy wells where a potassium ion could bind during its progress through the channel. With single file movement, diffusion through the pore has to be highly co-ordinated.

ii. Chloride Channels. Boyle and Conway (1941) first showed that skeletal muscle was permeable to chloride and Hodgkin and

Horowicz (1959b) showed that both potassium and chloride could contribute to the membrane potential although the distribution of chloride was passive according to the dictates of the membrane potential. Sudden alterations in the chloride equilibrium by altering $[Cl]_o$ caused transient depolarizations which were reversed as the chloride was redistributed with a time constant of 4 minutes. Hodgkin and Horowicz (1959b) further calculated that the frog muscle membrane was twice as permeable to chloride than it was to potassium and the resting membrane conductance for chloride ($\bar{g}Cl$) was 0.2 $mScm^{-2}$.

Hutter and Padsha (1959), Hutter and Noble (1960), and Moore (1969) obtained similar results when $[Cl]_o$ was replaced by impermeant anions and Hutter and Noble (1960) found that chloride contributed 68% of the total resting membrane conductance. Membrane permeability to chloride was thought to be little affected by potential. However later work (Hutter and Warner, 1972; Warner, 1972) demonstrated that chloride conductance varied with time, pH and membrane potential.

Adrian (1961) measured ^{36}Cl fluxes and estimated that the intracellular concentration of chloride was consistent with a purely passive distribution of chloride. However the fluxes of chloride across the membrane were found not to be independent. Based on this, and the fact that the channels showed anomalous mole-fraction dependence of conductance i.e. membrane resistance was increased more than expected on the basis of independence (Hutter and Padsha, 1959), Hille and Schwarz (1978) concluded that chloride channels were multi-ion pores.

Hutter and Warner (1967a, 1967b, 1967c) studied the pH dependence of the chloride channel and concluded that the membrane permeability for

chloride was increased in alkaline pH, but reduced in acid pH. Three divalent cations, Cu^{++}, Zn^{++}, UO_2^{++}, were also found to reduce membrane permeability to chloride. This finding was taken to suggest that these ions could compete with hydrogen for sites on a moiety which controlled permeability. The evidence implicated the charge on an imidazole group since the pH-chloride reaction had a close to neutral pK and the site was extremely sensitive to Cu^{++} and Zn^{++} both of which readily accept electrons from imidazole. Other possible negatively charged groups (eg. carboxyl, phosphate) were excluded on the basis of the lack of reactivity to other ions.

Woodbury and Miles (1973) investigated the permeability of the chloride channel to a wide variety of anions. They discovered that there were apparently two classes of anions. One type was classified as chloride-like since they showed the same pH dependence i.e. a decrease in conductance with decreasing pH. These were Cl^-, Br^-, NO_3^-, I^-, trichloroacetate, lactate, benzenesulphonate, isethionate, methysulphonate, glutamate, cysteate. The other ions were found to have conductances which increased as pH decreased and which were irreversible. These were termed benzoate-like anions i.e. benzoate, valerate, butyrate, proprionate, formate, acetate. The possibility that the two different types of pH-conductance relation were due to permeation through different channels was tested by the use of Zn^{++}.

Zn^{++} had been found to reduce the membrane conductance to chloride (Mashima and Washio, 1964; Hutter and Warner, 1967c). The effect of Zn^{++} on both benzoate and chloride was tested with the assumption that Zn^{++} would only affect the conductance to benzoate if it was using the same

channel as chloride. Zn^{++} was found to block both chloride and benzoate conductance equally at the different concentrations used and it was therefore concluded that the two types of anion were permeating through the same channel.

From their data, Woodbury and Miles (1973) developed a model of the anion channel in frog muscle based on that proposed by Hutter and Warner (1967b, 1967c). They calculated that the minimum dimensions of the channel were 5.5 x 6.5 Å based on the dimensions of the largest permeating anions (trichloroacetate, benzoate, benzenesulphonate). The channel was assumed to be thin in the sense that the rate of passage of an anion through the channel depended only on the rate of passage at one site which was the rate limiting step. The permeability and conductance sequence was that expected for a thin membrane with neutral (dipolar) sites (Barry and Diamond, 1971). This step was located near a group which was dipolar at high pH and which was capable of accepting a proton - the reaction having a pKa of 7. Hutter and Warner (1967c) had previously postulated that this group was imidazole. Woodbury and Miles (1973) further suggested that since the permeability of the benzoate-like anions was directly related to their hydrophobicity, then there was a hydrophobic site adjacent to the rate limiting step. The rate limiting step was postulated to be an energy well (binding site) for the chloride-like anions which could then interfere with the passage of other ions through the channel (Hutter and Padsha, 1959). However this step was suggested to be an energy barrier for the benzoate-like anions which would therefore not remain in this region of the channel and would not interfere with the passage of other ions. Normally these anions

would bind preferentially to water rather than to an imidazole dipole because the carboxyl group has a high affinity for H^+ (pKa's 4-5). Therefore it would be energetically unfavourable for these ions to come out of solution and enter the channel so the conductance to these ions would be low at normal pH. As pH is lowered, the protonation of the imidazole group would increase its field strength and thus lower the energy barrier to these anions. The conductance sequence would therefore be expected to be the reverse of the pKa sequence i.e. a high pK means that an anion would have a high affinity for H^+, a high field strength, a high potential energy barrier and therefore a low conductance. This was not the case however, and it was suggested that due to the good correlation between hydrophobicity and conductance, the binding to a hydrophobic site reduced the height of the adjacent energy barrier. Benzoate-like anions with sufficiently large hydrophobic groups were postulated to bind to channel sites and this was substantiated by the experiments of Bryant and Morales-Aguilera (1971) and Palade and Barchi (1977b). They found that such like anions eg. anthracene-9-carboxylic acid did indeed reduce membrane chloride conductance.

Estimates of chloride conductance ($\bar{g}Cl$) have been made in amphibian and mammalian muscle. In frog $\bar{g}Cl$ was found to be about 0.2 $mScm^{-2}$ and contributed about two thirds of the total membrane conductance (G_m) (Hodgkin and Horowicz, 1959b; Mashima and Washio, 1964; Stanfield, 1970; Fink and Lüttgau, 1976). $\bar{g}Cl$ for goat muscle was found to be 0.6 and 0.8 $mScm^{-2}$ which was 83% of G_m (Bryant and Morales-Aguilera, 1971; Bryant and Camerino, 1976). Estimates of $\bar{g}Cl$ for rat varied between 0.9 and 3.2 $mScm^{-2}$ (Camerino and Bryant, 1976; Lorković and Tomanek, 1977;

Palade and Barchi, 1977a) but was found to contribute 85-90% of the total membrane conductance. In their study on adult avian muscle, Morgan *et al.* (1975) found that $\bar{g}Cl$ was 2.6 $mScm^{-2}$ for the pigeon which was 83% of the total membrane conductance. The rest of the membrane conductance in all cases was attributed to potassium as it was assumed that any contribution from sodium was negligible.

The distribution of chloride has been believed to be a passive process since the experiments of Hodgkin and Horowicz (1959b) on frog muscle. However Hutter and Warner (1967a) found that there was evidence of active movement of chloride when the chloride conductance was reduced by acid pH. With the advent of ion sensitive electrodes, direct measurements of the internal activity of chloride ions could be made and it was found that this was higher than expected if chloride was passively distributed (Kernan *et al.*, 1974).

Bolton and Vaughan-Jones (1977) extended this observation to experiments in which continuous measurement of the chloride activity were made and found that E_{Cl} differed from E_m at equilibrium. They confirmed the suggestion by Hutter and Warner (1967a) that under conditions of low pH where the membrane permeability to chloride was low, then the muscle fibres actively accumulated chloride. Active accumulation of chloride ions has been reported for other systems eg. squid giant axon (Keynes, 1963; Russell, 1976) and smooth muscle (Casteels, 1971) and cardiac muscle (Lamb, 1961; Ladle and Walker, 1975). The resting permeability to chloride is much lower in these situations than occurs in the frog muscle. Bolton and Vaughan-Jones (1977) suggested that the apparent passive distribution of chloride in frog muscle reflected the high

permeability. Dulhunty (1978) also suggested that an active influx of chloride could occur in certain mammalian skeletal muscles *viz.* mouse extensor digitorum longus and soleus muscles; rat sternomastoid muscle but not in rat diaphragm or apparently, goat intercostal muscles (Bryant and Morales-Aguilera, 1971). This suggests that chloride can influence membrane potential of muscle fibres.

iii. Sodium Channels. The resting muscle membrane is permeable to sodium, although this permeability is low. The permeability ratio P_{Na}/P_K was calculated to be 0.013 from the data of Hodgkin and Horowicz (1959a) and the resting membrane conductance of sodium in squid axon was found to be 1 μScm^{-2} (Narahashi and Seyama, 1974). Several toxins were discovered to increase the resting sodium permeability in excitable membranes which resulted in depolarization and repetitive firing of action potentials either spontaneously or after a single stimulus. These toxins are: batrachotoxin (BTX) (from the Columbian arrow poison frog *Phyllobates aurotaenia*; (Albuquerque *et al.*, 1971a; Narahashi and Deguchi, 1971); grayanotoxins-I, -II, -III (GTX) (toxic principles from the leaves of Ericaceae, *Rhododendron* and *Andromeda*-species (Deguchi and Sakai, 1967; Seyama and Narahashi, 1973; Narahashi and Seyama, 1974); veratridine (an alkaloid from the plants of the families *Veratreae*, *Liliaceae* and *Melanthioideae*) (Frank, 1958; Shanes, 1958; Ulbricht and Flacke, 1965; Catterall and Nirenberg, 1973); aconitine (an alkaloid obtained from *Aconitum napellus*) (Peper and Trautwein, 1967; Schmidt and Schmitt, 1974; Catterall, 1975b); scorpion toxins from the venom of *Leiurus quinquestriatus* (Adam *et al.*, 1966; Koppenhoffer and Schmidt, 1968); *Tityus serrulatus* (Gomez *et al.*, 1973; Warnick *et al.*, 1975) and

Buthus tamulus (Narahashi *et al.*, 1972); and ciguatoxin from ciguatenic moray eels (Rayner, 1972).

These effects could all be prevented by the concomitant application of tetrodotoxin (TTX) or by the removal of external sodium ions. The action of BTX was irreversible and was not prevented when lithium replaced sodium as the major external cation (Albuquerque *et al.*, 1973). This suggested that the toxins were acting at the site of sodium permeation rather than with the ions themselves. The action of TTX was non-competitive (Albuquerque *et al.*, 1976; Seyama and Narahashi, 1973). Experiments with squid axons demonstrated that most toxins were more effective on internal application (Meves, 1966; Narahashi and Deguchi, 1971; Ohta *et al.*, 1973; Narahashi and Seyama, 1974; Schmidt and Schmitt, 1974; Catterall, 1975b; Warnick *et al.*, 1975).

Narahashi (1975) considered that since the effect of TTX was non-competitive and that the permeability ratio of squid axons to various inorganic and organic cations was different in GTX-I treated axons (Hironaka and Narahashi, 1975) compared to the permeability ratio found in the frog node of Ranvier during activity (Hille, 1971, 1972) there were two separate channels for the passage of sodium ions. One was opened by an action potential and blocked competitively by TTX, the other was open at rest and this was the channel at which toxins acted to increase resting sodium permeability. TTX was a non-competitive blocker of this channel. However Catterall and Nirenberg (1973) found that neuroblastoma cell lines incapable of generating action potentials were unaffected by veratridine. Catterall (1975b, 1975c) later showed that BTX, veratridine and aconitine all acted at the same site and competed with each other. This favours the idea that in resting membranes,

sodium permeates through the channels which are activated by depolarization. TTX itself has been found to reduce resting sodium permeability. A small hyperpolarization was found in giant axons in the presence of TTX (Freeman, 1969; Narahashi, 1972). TTX did not affect ouabain sensitive efflux of sodium which implied that TTX does not affect electrically neutral sodium exchange but inhibits only passive inward sodium movements (Freeman, 1971; Baker *et al.*, 1969). This effect of TTX was abolished in sodium free media but was unaffected if sodium was replaced by lithium (Albuqerque *et al.*, 1973). In skeletal muscle, the action of BTX was blocked by TTX, indicating that here also the sodium conductance which is activated by an action potential occurs at the same channel through which sodium permeates in the resting state (Albuquerque *et al.*, 1971b).

Further work by Catterall and co-workers (Catterall, 1975b, 1975c, 1976, 1977a, 1977b, 1979; Catterall and Ray, 1976; Ray and Catterall, 1978; Catterall and Morrow, 1978) revealed two separate binding sites; one to which BTX, GTX, veratridine and aconitine bound and one to which the toxins from scorpion venoms bound i.e. *Leiurus quinquestriatus* (Okamoto *et al.*, 1977) and *Androctonus australis Hector*(Couraud *et al.*, 1978; Catterall and Beress, 1978). Couraud *et al.* (1978) found that the toxin isolated from the sea anemone, *Anemonia sulcata* also bound to this site. The two sites were postulated to be allosterically coupled and to exhibit positive heterogenic co-operativity since the binding of a toxin at one site reduced the apparent dissociation constant for a toxin binding at the other site. Catterall (1975b) proposed that binding of toxin at one regulatory site at the sodium channel would alter the equilibrium between closed and open channels in favour of the open state. When a second

toxin was bound at the second site the equilibrium was shifted further towards the open state. TTX and a related toxin saxitoxin, (STX) obtained from the dinoflagellate *Gonyaulax catanella* and concentrated in shellfish, were non-competitive inhibitors of the action of BTX, aconitine and veratridine. Similarly TTX was not competitive with *Leiurus* toxin indicating that both regulatory sites were separate from the site at which TTX and STX acted to prevent sodium influx. Catterall and Ray (1976) found that the dissociation constant for binding of *Leiurus* toxin was increased by depolarization and concluded that the scorpion toxin binding site was located on a voltage sensitive regulatory component of the ion channel. The binding of scorpion toxin inhibited the inactivation of sodium conductance during an action potential (Catterall and Morrow, 1978; Catterall, 1979). The voltage dependence of scorpion toxin binding however had the same kinetics as those of activation. Catterall (1979) postulated that scorpion toxin bound to a region of the sodium channel involved in voltage dependent activation but then interfered with the coupling of activation to inactivation thus causing a slowing of the kinetics of inactivation. The kinetics of the sodium and potassium channels activated during an action potential will now be discussed.

2. Transient Channels

i. Hodgkin-Huxley Kinetic Model. Hodgkin, Huxley and Katz (1952) developed a method for controlling the membrane potential of an area of squid axon membrane based on the work of Cole (1949) and Marmont (1949). In this voltage clamp technique, the membrane potential is forced to follow prescribed step changes by a negative feedback amplifier

which passes the necessary current across the membrane. The size and direction of the current flow is used to investigate ionic conductance changes during the voltage step. The membrane current consists partly of capacity current and partly of ionic current under normal circumstances. With the voltage clamp the membrane capacity is charged at a constant level for most of the time and the majority of the observed current is ionic. Because each ion tends to move passively down its electrochemical gradient in accordance with the constant field equation, this can be used to predict whether the net movement of an ion is inward or outward at a given potential. Therefore if the membrane potential is clamped at the equilibrium potential of a particular ion, this ion will contribute nothing to the membrane current. In addition since the permeability changes are relatively insensitive to the concentration of the major ions, the movements of one ion can be stopped by replacing it with an impermeant species with little change in the movement of other ions.

Hodgkin, Huxley and Katz (1952) found that there were several stages in the development of the ionic current following a step in potential from E_m to 0 mV. Firstly there was a brief surge of capacity current associated with the sudden change in membrane potential followed by an ionic current during the period the potential was maintained at 0 mV. The ionic current was initially inwardly directed but this changed fairly rapidly into a large prolonged outward current. The phase of inward current disappeared at about +50 mV and was replaced by one of outward current. When sodium was replaced by choline and equilibrium potentials were measured, Hodgkin and Huxley (1952a, 1952b) separated the ionic current into three components; the early inward current carried by

sodium, the late outward current carried by potassium and a small time-invariant component carried by unidentified ions termed the leakage current. The sodium current rose rapidly to a peak but thereafter declined to negligible values after a few milliseconds whereas the potassium current rose more slowly to a steady value. Hodgkin and Huxley (1952c) then showed that sodium conductance was turned off by an inactivation process which was potential dependent. In the final paper of the series, Hodgkin and Huxley (1952d) described a mathematical model which would account for their findings and which would also account for the conduction and excitation in quantitative terms. The total ionic current was split into its constituent components,

$$I_i = I_{Na} + I_K + I_l$$

I_i = total ionic current
I_{Na} = current due to Na^+
I_K = current due to K^+
I_l = current due to other ions

They found that the relationship between current and voltage at any instant was linear as would occur in an ohmic conductor and therefore the component ionic movements were related to the driving forces by an expression akin to Ohm's law. These were

$$I_{Na} = gNa(E - E_{Na})$$

gNa = sodium conductance
E = membrane potential
E_{Na} = equilibrium potential for sodium

Similarly $I_K = gK(E - E_K)$

$$I_l = \bar{g}l(E - E_l)$$

The value $\bar{g}l$ is constant since it does not vary with time or voltage. However the conductances for sodium and potassium did vary. Although the degree and rate of activation of gNa and gK increased with increasing

depolarization, they never exceeded a maximum (represented by $\bar{g}Na$ and $\bar{g}K$). An increasing fraction of the maximum permeability was activated by increasing depolarization. This was described mathematically:

$$gNa = m^3 h \bar{g}Na$$

$$gK = n^4 \bar{g}K$$

where m^3h and n^4 represented the fractions of the sodium and potassium permeabilities which were turned on. The coefficients h, m and n are dimensionless parameters which vary between 0 and 1 with first order kinetics and rate constants which depend on the potential. They are continuous functions of potential and time. The sigmoid shape of the rising phase of permeability observed after a depolarizing step was obtained by raising the m and n variables to higher powers.

At any membrane potential the values of h, m and n relaxed towards steady state values h_∞, m_∞ and n_∞ along an exponential time course of time constant τ_h, τ_m and τ_n. At the resting potential m_∞ and n_∞ were small and h_∞ was large. Therefore the resting conductances to sodium and potassium are small at rest. After a depolarization, m_∞ would rise rapidly thereby activating the sodium conductance but h_∞ would start decreasing at the same time and the sodium conductance would become inactivated. The potassium conductance would be activated by a slow increase in n. The three parameters were dependent on the membrane potential but not on each other.

Hodgkin and Huxley (1952d) then found that this model simulated the form, amplitude and threshold of the propagated action potential recorded in squid axons, the refractory period and sub-threshold responses. They also considered the physical basis of the model. The alterations in

membrane conductance were potential but not membrane current dependent and they suggested that the conductance changes arose from the effect of the membrane electric field on the distribution or orientation of molecules with a charge or dipolar moment. An earlier theory (Hodgkin, Huxley and Katz, 1949) in which sodium ions traversed the membrane in combination with a lipoid soluble carrier which was negatively charged was rejected. The first effect of depolarization in this model would require a movement of the negatively charged carrier molecules from the outside to the inside of the membrane i.e. and initial outward current. This was not observed under conditions of voltage clamp. They then postulated that the movement of sodium was dependent on the distribution of charged particles which did not act as carriers but allowed the passage of sodium when they occupied particular sites in the membrane. The rate of movement of the activating particles (m parameters) determined the rate at which the sodium conductance approached the maximum but it had little effect on the magnitude of the conductance. The transient nature of the sodium conductance was attributed to the relatively slow movement of another particle (h parameter) which blocked the flow of sodium ions when it reached a certain position in the membrane.

ii. Gating of Sodium Channels. Hodgkin and Huxley (1952d) proposed that the potassium and sodium pathways were separated, because the kinetics were so different. There was no simple way of converting the selectivity of the particles for sodium to a selectivity for potassium. They predicted that the movement of the hypothetical gating particles would cause an outward flow of current accompanying the sodium current. They also stated that the density of these components was

relatively low and that a number of sodium ions crossed the membrane at a single active patch otherwise this outward current would be of the same magnitude as that observed for sodium.

This theory was later modified for other situations; i.e. at the frog node of Ranvier gNa was more closely approximated by m^2h and gK by n^2k where k was an inactivation parameter with an extremely long time course (many seconds) (Frankenhaeuser, 1960, 1963). Frog muscle was found to obey the original Hodgkin-Huxley formulation by Dodge (1961) although for very long depolarizations a parameter for potassium inactivation was required. If the membrane was strongly hyperpolarized before depolarization the opening of the ion channels was delayed (Cole and Moore, 1960; Keynes and Rojas, 1976; Neumcke *et al.*, 1976). This could not be accounted for by the m^3h and n^4 parameters and higher powers of the activation parameters were required to fit the data. Further deviations from the Hodgkin-Huxley model were found when the kinetics of the charge movement associated with the gating of the ion channels were investigated (Ulbricht, 1977).

The current associated with the movement of the charged particles described by the Hodgkin-Huxley kinetic model was detected only relatively recently by Armstrong and Bezanilla (1973a, 1973b) and Keynes and Rojas (1973). Ionic current was suppressed by replacing the permeant ions Na^+ and K^+ in both the external and internal solution with TRIS for external Na^+ and Cs^+ for internal K^+. TTX was also used to block any residual current through the Na^+ channel in some experiments. Gating current was unaffected by TTX. The linear portion of the remaining capacity current was eliminated by algebraically summing the current from a

positive step to the current from a negative step of equal magnitude. Any capacitative current remaining after this procedure would be non-linear and hence associated with gating current since gating current would only flow for positive voltage steps but not for negative voltage steps. Gating current I_g was found to rise rapidly to a peak which then decayed monotonically as would be expected of the current produced by the reorientation of dipolar molecules in a suddenly altered field. The majority of the current was over before the sodium channels opened as would be expected if this charge movement reflected gating.

Further evidence that this non-linear capacity current was associated with gating was obtained by using procedures which affect sodium current (Armstrong and Bezanilla, 1973a, 1974, 1975; Bezanilla and Armstrong, 1974, 1975). Gating current did not have an equilibrium or reversal potential and the charge movement during turn-on as the gates opened was equal and opposite to the charge movement turn-off as the gates closed. Most of the internal and external ions in the squid axon could be replaced without altering gating current; all the above indicating that gating current was capacitive in origin. The time course of the non-linear current was almost identical to that for I_{Na} at the pulse end when the gates were turning off. Internal perfusion with $ZnCl_2$ which had previously been shown to block I_{Na} (Begenisich and Lynch, 1973), inactivation by a short prepulse and prolonged depolarization (Bezanilla and Armstrong, 1974) were all found to decrease gating current reversibly. Both I_{Na} and I_g were reduced proportionately and they recovered in parallel. Internal treatment of axons with a proteolytic enzyme mixture, pronase,was found to remove sodium inactivation without

affecting Na or K activation kinetics (Armstrong *et al.*, 1973). Similarly treatment with pronase removed I_g inactivation (Armstrong and Bezanilla, 1974).

Bezanilla and Armstrong (1975) found that there was a rapidly rising phase of gating current which meant that there was relatively little charge movement associated with the initial steps involved in opening a channel, and according to the Hodgkin-Huxley m^3 formulation this was predicted to be an exponentially decaying current. There were two phases of decay of gating current associated with a step depolarization. The rapid phase was clearly associated with opening of the Na^+ channels but the slower phase was less easy to interpret. Originally they postulated that this phase was associated with the K^+ channels (Bezanilla and Armstrong, 1975). However with better records the kinetics of the slow phase were too fast to arise from potassium channels and none of the known properties of the sodium channel correlated with this component. The kinetics were faster than those for sodium inactivation, and they could only speculate that maybe it represented an event which was a necessary prelude to inactivation.

A sequential scheme for activation explained the kinetics of opening of Na^+ channels i.e. 3 m particles were required to be in the permissive state before a channel would open but closing of Na^+ channels was a first-order process i.e. 1 h particle moved from a permissive state to close the channel. However sequential kinetics predicted that gating current would have a time course three times longer than that of I_{Na}. Channel closure would be complete after one charge movement but gating current would continue to flow until all channels were in the resting

state. Gating current was found to inactivate in parallel with I_{Na}. Bezanilla and Armstrong (1975) therefore suggested that channel opening occurred by one path and that channel closing occurred by a different path. This was supported by the finding that after a long pulse the charge movement of the tail of gating current was only a third of that involved in activation. The other two thirds appeared to return to the resting position at about the same rate that I_{Na} recovered from inactivation. Perfusion with pronase prevented the development of this reduction in charge movement.

The Hodgkin-Huxley equations predicted that the kinetics of activation and inactivation were independent. However the above data do not support this and suggest rather that the kinetics of activation and inactivation are coupled.

Hoyt (1963) analysed some of the data from Hodgkin and Huxley's axon 17 in terms of a special case of a coupled activation-inactivation model and later Hoyt (1968) found that her kinetics predicted a different result from those of Hodgkin and Huxley (1952d). The steady state inactivation h_∞ curve should be nearly invariant as the potential selected for the test step was altered in H-H kinetics. In Hoyt's model this curve was predicted to be shifted to the right along the voltage axis as the test step was changed to one during which the peak gNa was greater. Hoyt and Adelman (1970) did find such a shift but the effects were marginal. The results could also be produced by a membrane obeying H-H kinetics in the presence of an uncompensated series resistance (Goldman and Schauf, 1972). However Goldman and Schauf (1972) repeated the experiment and found the predicted shift of h_∞. This was more direct evidence that

the amount of inactivation produced by a conditioning depolarization could affect the degree of activation of sodium conductance during a subsequent test depolarization.

More recently, Bezanilla and Armstrong (1977; Armstrong and Bezanilla, 1977) have investigated the coupling of activation to inactivation. They concluded that the transition of the sodium channel between the open and inactivated state was voltage independent. This would account for the absence of a component of gating current with h kinetics. However inactivation was voltage-dependent as a consequence of coupling to the activation process i.e. the activation 'gate' of the sodium channel was required to be open before the inactivation 'gate' could close. From their voltage clamp data they described a second open state for the sodium channel. This is at variance with the observations of Sigworth (1977) in frog node of Ranvier. Using noise analysis he concluded that sodium channels existed in two conductance states - open or closed.

Armstrong and Bezanilla (1977) proposed a multi-state model for the sodium channel. Several other multi-state kinetic models had been proposed previously (Hoyt, 1963; Goldman, 1964; Moore and Jakobsson, 1971; Goldman, 1975). Chandler and Meves (1970a) and Chiu (1976) have proposed a multi-state model for inactivation and there is also some evidence that the kinetics of the potassium conductance may be similarly controlled (Frankenhaeuser, 1963; Palti *et al.*, 1976; Fishman *et al.*, 1976). None of the kinetic models proposed has yet described all the features of the sodium conductance, and Hille (1978) has suggested that studies of artificial membranes which have been doped with channel forming peptides or proteins is useful. Such membranes can imitate phenomena

in nerve closely and have the advantage of being completely defined. The chemical requirements for gating can then be investigated systematically. Armstrong and Bezanilla (1977) postulated that inactivation involved the movement of an endogenous blocking particle into the sodium channel from the axoplasmic site, thus stopping ion flow as well as hindering subsequent conformational changes. This latter effect was termed charge immobilization. Under maintained depolarization, the voltage-sensitive portions of the sodium channel are partially immobilized so that the full gating current cannot be produced during subsequent potential steps. Cahalan and Almers (1979a, 1979b) investigated whether two of the small molecules which can block sodium channels from the inside (a quaternary derivative of lidocaine, QX-314 and N-methylstrychnine) could also produce charge immobilization. Both did indeed in a manner very similar to the physiological process. Cahalan and Almers (1979a, 1979b) suggested in fact that the local anaesthetic stabilized the association between the channel and the endogenous inactivating particle, whereas the N-methylstrychnine competed with this hypothetical particle for its binding site. Pancuronium has also been suggested to compete with the blocking particle in a manner similar to N-methylstrychnine (Yeh and Armstrong, 1978).

Various treatments have been found to affect the kinetics of activation or inactivation of the sodium channel. Those treatments which were only active on internal perfusion of the squid axon or after the application of a depolarizing stimulus with no effect on the kinetics of activation were regarded as specifically affecting inactivation (h process) eg. pronase (Armstrong *et al.*, 1973), the toxic principle of the

sea anemone *Condylactis gigantea* (Narahashi *et al.*, 1969), fluoride (Chandler and Meves, 1970b), *Leiurus* toxin (Koppenhoffer and Schmidt, 1968). An effect on the kinetics of activation with no apparent effect on inactivation has been found for the venom of the North American scorpion *Centuroides sculpturatus* (Cahalan, 1974). Catterall (1979) suggested that *Leiurus* toxin and *Centuroides* venom bound to the same site in the membrane but that *Leiurus* interfered with the coupling of activation and inactivation whereas *Centuroides* interfered with activation only. Because inactivation was susceptible to internal protease treatment, the gate was postulated to be a readily accessible protein attached to the inner end of the sodium channel. The other treatments, which were effective both externally and internally, *viz*. BTX, (Albuquerque *et al.*, 1971a, 1971b; Narahashi and Deguchi, 1971) GTX, (Deguchi and Sakai, 1967; Seyama and Narahashi, 1973; Narahashi and Seyama, 1974) veratridine (Shanes, 1958; Ulbricht and Flacke, 1965; Catterall and Nirenberg, 1973); aconitine (Peper and Trautwein, 1967; Schmidt and Schmitt, 1974; Catterall, 1975b) could therefore be deduced to open the m^3 activation gate and then prevent the inactivation process.

TTX and STX abolished the sodium current without affecting gating current, potassium current, or the binding of many of the above toxins to their respective binding sites at the sodium channel. They also abolished the transient increase in conductance to other ions which could permeate the sodium channel and therefore TTX and STX were concluded to block the channel rather than to interact with the permeating ions (Tasaki and Hagiwara, 1957; Hille, 1968a; Moore *et al.*, 1967a; Binstock and Lecar, 1969). Narahashi *et al.*, (1967) found that TTX was

ineffective when perfused internally in the squid giant axon, suggesting that it bound to a site on the exterior of the membrane. The binding sites for the toxins and the activation and inactivation 'gates' are therefore thought to be located deep in the membrane beyond the TTX binding site.

Stallcup (1977) used the known interaction between veratridine and scorpion venom from *Tityus serrulatus* to separate the sodium channels in nerve and muscle cell lines into three categories. Scorpion venom potentiated the action of veratridine in the nerve cell lines by lowering the affinity constant (K_m) for veratridine without affecting the maximum rate (V_{max}) of sodium uptake observed with veratridine alone or by reducing K_m and increasing V_{max}. In muscle cultures scorpion venom increased V_{max} of sodium uptake without affecting K_m for veratridine. The types of cells used also varied in their affinities for TTX and scorpion venom. The ratio for TTX was mouse nerve > rat nerve > mouse muscle > rat muscle as expected from results *in vivo*. For the scorpion venom however, mouse nerve > mouse muscle > rat nerve and muscle. The significance of these results is unclear and they are open to the criticism that clonal lines have altered ion channel properties from the original cells. L_6 clonal line has altered electrophysiological properties from primary rat muscle cultures e.g. L6 myoblasts have a resting membrane potential of -60 to -70 mV and this does not alter during maturation. The specific membrane resistance is very high and the fibres are relatively impermeable to both sodium and chloride (Kidokoro, 1973; 1975a; 1975b). The myoblasts also show unusual responses to cholinomimetics (Steinbach, 1975).

The permeability of the sodium channels to a variety of inorganic and organic cations has been measured (Chandler and Meves, 1965; Binstock and Lecar, 1969; Moore *et al.*, 1966; Hille, 1971, 1972; Binstock, 1976). On the basis of these permeability studies, Hille (1971, 1972, 1975a) proposed a model of the sodium channel in which the narrowest portion of the pore, the selectivity filter was about 3 x 5 Å. All the permeant ions could fit this on the basis of unhydrated radii. However several impermeant ions, notably methylamine could also fit into a pore this size. Methylamine is nearly isosteric with hydroxylamine and hydrazine which were much more permeable and it is much smaller than formamidine or guanidine which both penetrated the channel. Hille (1971, 1972) proposed that the selectivity filter was lined with oxygens which could form hydrogen bonds with amino and hydroxyl groups on the permeant cations thus reducing their effective diameter to 3.1 Å. The methyl groups are incapable of hydrogen bonding and would therefore be excluded since they are 3.8 Å wide. The passage of metal cations was accompanied by some water of hydration and the observed permeability sequence followed that expected for binding to a high field strength anion (Eisenman, 1962), i.e. they deviated markedly from the independence principle. The independence principle assumed that the movement of an individual ion was independent of the movement of every other ion. The P_{Na}/P_K ratio was found to depend on the internal potassium concentration (Chandler and Meves, 1965). Deviations were found for other cations (Hille, 1975a) and Hille and Schwarz (1978) considered that the sodium channel showed some hint of a multi-ion pore.

Huang *et al.*(1979) have determined the permeability sequence in BTX

activated channels of two nerve cell lines in culture. One line was TTX sensitive and one was relatively resistant. The permeability sequence for inorganic and organic cations was identical and H^+ competed with Na^+ and TTX at the channels with a pKa of 5.5. The limiting size of the BTX activated channel was calculated to be 3.8 x 6.0 Å since methylamine was permeable. They concluded that BTX altered the conformation of the selectivity filter to allow larger ions to permeate than normally occurs in potential activated channels.

Hille (1975a) later considered a four barrier energy model of the selectivity filter. Eight oxygens, six of them in approximately one plane were the donors for hydrogen bonding. Two of these oxygens were proposed to belong to an acid carboxyl group, the protonation of which leads to block of the channel. The high field strength anion binding site was proposed to be at this moiety. Initially a cation was postulated to be fully hydrated but as it approached the carboxylic acid oxygen of the selectivity filter, the ion would lose a small number of water molecules but be stabilized by its attraction to the negatively charged oxygen. This was the bound state. As the ion entered the selectivity filter, it was still in contact with the oxygen, but was required to lose many more water molecules thus forming a high energy transition complex. The energy of this complex would depend on the ion species and this therefore would determine channel selectivity. The ion would then move on through the narrow region and regain some of its water of hydration. Finally the ion would reach the axoplasm over energy barriers no higher than those for aqueous diffusion. The dimensions of the selectivity filter are such that an accompanying water molecule could hydrogen-

bond to an oxygen on the other side of the pore.

Hille (1975b) further proposed a model for the TTX and STX binding site. Both toxins contained a guanidinium moiety and since free guanidinium could penetrate the channel (Hille, 1971) he proposed that the toxins stuck in the selectivity filter. TTX and STX binding was inhibited by the cations which had been proposed to bind to the carboxyl group of the filter *viz.* calcium and thallium (Henderson *et al.*, 1974). Hydrogen bonding between five of the pore oxygens and portions of the toxin molecule and the electrostatic attraction between the guanidinium group and the negative charge of the selectivity filter would stabilize the complex. STX is a rigid molecule possessing two guanidinium groups but only one (around C_8) protruded enough to be inserted into the selectivity filter. To accommodate both molecules the entrance to the selectivity filter was suggested to be 9 x 10 Å in area before narrowing to 3 x 5 Å at the filter itself. The effect of TTX is reduced in acid and alkaline solutions. High pH would convert TTX into the inactive zwitterion and the effect of low pH was best described by a competition between TTX and H^+ for the same receptor i.e. the carboxyl of the selectivity filter (Ulbricht and Wagner, 1975). Reed and Raftery (1976) showed that methylation of a carboxyl group blocked TTX binding to the sodium channel. This indicated that there may be a carboxyl group which is important for TTX binding but which did not appear to be located at the selectivity filter. The methylated channel had normal ion selectivity and was blocked by H^+ with a pKa of 5.2 as in normal channels (Spalding, 1978).

Because TTX and STX block sodium channels with high specificity,

they have been used to estimate the density of sodium channels in excitable membranes. Concentration-effect curves indicated that a single toxin molecule bound to each channel (Hille, 1968a). The earliest attempts to estimate sodium channel density used a bioassay to estimate the amount of TTX taken up by a nerve membrane at the time of block and hence sodium channel density (Moore *et al.*, 1967b). Moore *et al.* calculated that there were fewer than 13 sodjum channels per μm^2 of lobster axon. Subsequently Keynes *et al.* (1971) confirmed the sparseness of TTX binding sites in the lobster, crab and rabbit nerve. Later studies were performed with radioactively labelled toxin. Levinson (1975) however pointed out the difficulty in assessing the purity of the labelled toxin even when it was apparently radiochemically pure. By comparing the saturable uptake of toxin by bioassay with the saturable uptake of radioactivity, the purity of his preparation was only 0.3. This means that channel density could be grossly underestimated, and this was what occurred in the early estimates of channel density (Colquhoun *et al.*, 1972; Henderson *et al.*, 1973).

With the development of an improved technique for labelling toxin (Ritchie and Rogart, 1977c) the saturable component of binding was totally sensitive to displacement with excess toxin. Channel density in small non-myelinated fibres was estimated as $35/\mu m^2$ for garfish olfactory nerve, $90/\mu m^2$ for lobster walking leg nerve and $100/\mu m^2$ for rabbit vagus (Ritchie *et al.*, 1976). Ray *et al.* (1978) calculated that the density of sodium channels on synaptosomes was 35 sites/μm^2 as estimated by binding of scorpion toxin. This was similar to that found on unmyelinated nerve fibres. In squid axon, channel density was higher $550/\mu m^2$ (Levinson and

Meves, 1975) which was consistent with that estimated by Keynes and Rojas (1974) from the maximum charge movement associated with gating currents.

In myelinated nerve, where transmission of activity is saltatory, the number of toxin binding sites at a node was very high - 12,000/μm^2 in rabbit sciatic nerve (Ritchie and Rogart, 1977a) after allowance was made for binding to non-myelinated nerve. This corresponded to one channel for every 8000 $\mathring{A}^2$ of membrane. Estimation of channel density at frog nodes of Ranvier from noise measurement and gating current measurements gave values of 2,000/μm^2 and 5,000/μm^2 respectively (Conti *et al.*, 1976a, 1976b; Nonner *et al.*, 1975a, 1975b). Estimates of sodium channel density in muscle membrane were of the same order as found in squid axons i.e. 195 to 380 sites/μm^2 (Almers and Levinson, 1975; Jaimovich *et al.*, 1976; Ritchie and Rogart, 1977b).

These values for number of toxin binding sites did not take into account the possibility that sodium channels could occur on the T-tubular membrane. From electrophysiological data, Adrian and Peachey (1973) and Hille and Campbell (1976) inferred that 15 to 25% of the sodium channels were located in the T-tubular membrane although it has six times the membrane area of the surface. Jaimovitch *et al.* (1976) estimated the fraction of TTX binding sites on the surface membrane by using detubulated muscle. After glycerol treatment to seal off the T-tubular membrane more than 50% of toxin binding sites were inaccessible to the toxin. From this Jaimovitch *et al.* (1976) concluded that there were 175 sites/μm^2 on the surface membrane and 40-50 sites/μm^2 on the tubular membrane.

Solubilization of the TTX binding protein was achieved using Triton-X 100 (Henderson and Wang, 1972; Benzer and Raftery, 1973) and the

molecular weight was calculated to be about 500,000 daltons. Levinson and Ellory (1973) estimated the molecular size of the binding site by radiation inactivation and this gave a figure of 230,000 daltons which corresponded to a spherical protein 80 Å in diameter. Recently Agnew *et al.* (1978) improved the purification of this binding component by adsorption chromatography. The purified material had a sedimentation coefficient of 8S which correlated well with the estimate of molecular weight by Levinson and Ellory (1973). However the Stokes radius was 95 Å - unusually high for a 230,000 dalton component. SDS gel electrophoresis of the purified material gave 3 major polypeptide components of 46,000, 59,000 and approximately 300,000 daltons. Taylor (1978) has recently succeeded in extracting a proteoglycolipid from rat gastrocnemius which bound veratridine with a high specificity. The purified complex did not bind TTX or cholinergic ligands (α-BuTX, Ach, nicotine, d-TC, decamethonium, atropine, pilocarpine) and the veratridine binding was inhibited by aconitine but not by any of the above agents. The complex was found to be a proteoglycolipid with a protein : carbohydrate : phosphorus ratio of 1.5 : 1.1 : 1.0. Reconstitution of plasma membrane vesicles into liposomes with functional sodium channels has also been reported recently (Villegas *et al.*, 1977). These recent developments together with the synthesis of an affinity label derivative of TTX (Guillory *et al.*, 1977) are hopeful signs that the molecular composition of the sodium channel will soon be known in as great detail as that of the Ach receptor complex (for review see Heidmann and Changeux, 1978).

Single channel conductance (γ) estimates have been made for the sodium channel based on those of channel density together with experimental

values for membrane conductance and from noise analysis. In frog muscle, γ_{Na} was estimated as 5 pS for *Rana temporaria* (Almers and Levinson, 1975) and 8.5 pS for *Rana pipiens* (Hille and Campbell, 1976). In frog nodes of Ranvier, γ_{Na} was estimated as 8 pS by noise analysis (Conti *et al.*, 1976a, 1976b; Sigworth, 1977). Both found that γ_{Na} did not vary with voltage and Sigworth (1977) also found that treatment with TTX did not affect mean channel conductance. However γ_{Na} was reduced to 2.5 pS by lowering pH which was consistent with the blocking action of H^+ ions on channel conductance (Ulbricht and Wagner, 1975). Van den Berg *et al.* (1975) estimated that γ_{Na} in node of Ranvier was slightly lower at 2 to 5 pS. Conti *et al.* (1976a, 1976b) found that the frequency spectrum could be separated into 3 components. One of these components was 1/f noise which has a spectral density which is inversely proportional to frequency over a wide range of frequencies. This noise is associated with the movement of ions through open channels (Stevens, 1977). The other two components were the Lorentzian spectra usually encountered in noise analysis. The low frequency spectrum was identified as h gate fluctuations and the high frequency spectrum as m gate fluctuations (Conti *et al.*, 1976a, 1976b).

Estimates of mean channel conductance in squid axons were similar: 3 pS (Levinson and Meves, 1975) and 4 pS (Conti *et al.*, 1975). In cultured neuroblastoma cells, Ray and Catterall (1978) calculated that each sodium channel transported 1×10^8 ions min^{-1} and had a mean γ_{Na} of 3 pS. These values are fairly consistent but about an order of magnitude lower than estimate of single channel conductance through the Ach receptor complex (Neher and Stevens, 1977, Ulbricht, 1977).

iii. Potassium Channels. The potassium channels involved in the generation of the action potential, the delayed rectifiers, have been separated from the sodium channels on the basis of the kinetics (Hodgkin and Huxley, 1952b). Later tetraethylammonium (TEA) was found to eliminate the potassium current with little effect on the sodium current (Tasaki and Hagiwara, 1957; Armstrong and Binstock, 1965; Hille, 1967). On the basis of experiments with hydrophobic derivatives of TEA, Armstrong (1966, 1969, 1971, 1974) proposed a model of the potassium channel with a diameter of 8 Å at the entrance decreasing to a narrow portion of 3 Å diameter. The selectivity filter was suggested to reside in the narrow portion and the n^4 activation gate in squid axon was located on the inside of the membrane. However in frog node of Ranvier TEA blocked from the outside (Hille, 1967).

In the squid, Armstrong (1966, 1969, 1971, 1974) proposed that TEA and its derivatives could not block potassium currents unless the activation gate was open because of the time dependence of the block. In the frog node of Ranvier the external blocking site for TEA was apparently accessible regardless of whether the channel was open or closed (Armstrong and Hille, 1972). TEA also blocked resting potassium channels in skeletal muscle but the mode of blockade was non-competitive (Volle *et al.*, 1972). This difference between frog muscle and squid axon was attributed to either species difference or the external surface of muscle membrane had receptors for TEA which differed from those at the internal surface (Narahashi, 1974). *Aplysia* neurones have recently been found to possess several pharmacologically distinct potassium channels (Thompson,

1977; Klee, 1978) which may favour the first possibility. The channels were susceptible to different extents to TEA and a new potassium channel blocking drug, 4-aminopyridine (4-AP) (Pelhate and Pichon, 1974; Gillespie and Hutter, 1975; Bowman *et al.*, 1976; Schauff *et al.*, 1976; Yeh *et al.*, 1976a; Ulbricht and Wagner, 1976; Meves and Pichon, 1977; Kirsch and Narahashi, 1978). 4-AP blocked squid axons both on external and internal application (Yeh *et al.*, 1976a). Ulbricht and Wagner (1976) suggested that 4-AP acted from the inside even on external application in frog node of Ranvier. Therefore the possibility that the two drugs act on different receptors cannot be excluded.

A recent report by Wanke *et al.* (1979) has indicated that the activation gate of the potassium channel opened during an action potential has associated with it a proton-titratable group with a pKa characteristic of histidyl residues. When protonated, the channel conductance did not fall to zero and Wanke *et al.* (1979) proposed that for complete blockade a further more acidic side chain group must be protonated and suggested this was an imidazole group. Adrian and Peres (1977) have detected a component of capacity current in frog muscle which had kinetics of the n^4 activation gate of potassium channels. TEA did not affect this charge movement which supported Armstrong's hypothesis (1966, 1969, 1971, 1974) of an internally located gate. However far less is known about the structure of the potassium channel than the sodium channel.

The density of potassium channels on excitable membranes was not easy to estimate because of the lack of a drug with a high affinity for the channel. However recently Hucho (1977) has reported the development of a photo-affinity label derivative of TEA which bound relatively

specifically to the potassium channel of unmyelinated nerve fibres of crayfish with little effect on sodium channels. He estimated that the density of binding sites was 220/μm^2, but suggested that it was probably on the high side.

Potassium single channel conductance has been estimated from noise measurements, but the original estimate (Armstrong, 1975) was obtained from the rate of blockade of potassium channels by nonyltriethylammonium. Armstrong assumed that the entry rate of both ions into the channel was the same and calculated that γ_K was about 2 to 3 pS, but emphasized that this was probably on the low side. Begenisich and Stevens (1975) found that mean channel conductance was 4 pS and suggested that the channels only had two conductance states - open and closed. γ_K was independent of voltage. Siebenga *et al.* (1973) however found γ_K did exhibit some voltage dependence and ranged from 10 to 37 pS. Later they revised this estimate to 3 pS (van den Berg *et al.*, 1977). Conti *et al.* (1975) agreed with Begenisich and Stevens (1975) that γ_K was not voltage dependent and had a value of 12 pS. However this experiment was carried out on squid axon and the other experiments were performed with frog node of Ranvier which may account for the difference between the estimates. Single channel conductance in potassium channels is therefore similar to that found for sodium channels.

iv. Calcium Channels. Calcium has long been known to affect action potential generation. If the external concentration of calcium was increased then the critical depolarization required to reach firing threshold was also increased (Brink, 1954). In terms of the Hodgkin-Huxley model (1952d), calcium concentration changes shifted the

voltage dependence of activation and inactivation along the voltage axis as if the voltage sensing processes (i.e. the gates) experienced some hidden bias (Frankenhaeuser and Hodgkin, 1957). Gordon and Welsh (1948) postulated that the Ca^{++} cation was pulled into the membrane by the inwardly directed field at rest and released into the bathing medium as the inward field was reduced by depolarization i.e. the ions were supposed to plug the ionic channels acting simultaneously as the field sensor and gate. However no models based on this hypothesis agreed with experimental observations - in fact most contradicted this suggestion. Calcium ions could penetrate open sodium channels but not closed ones (Baker *et al.*, 1971b; Meves and Vogel, 1973). Excitability could be maintained for prolonged periods in solutions containing calcium chelating agents (Armstrong *et al.*, 1972) and in calcium free solutions (Frank and Inoue, 1973). The voltage dependence of opening of sodium channels remained as steep as in normal solutions (Hille *et al.*, 1975). Lastly a weakly voltage dependent block of sodium channels by calcium ions was found in frog nerve and muscle (Woodhull, 1973; Hille *et al.*, 1975). 10-33% of the channels were calculated to be blocked by this mechanism at rest, far too low to account for the extremely low permeability to sodium.

An alternative hypothesis (Frankenhaeuser and Hodgkin, 1957) that calcium was acting via effects on membrane surface charge was proposed and extensive testing has shown that this is the mechanism by which calcium affected gating (Gilbert and Ehrenstein, 1969; Mozhayeva and Naumov, 1970, 1972a, 1972b, 1972c; Brismar, 1973; Hille *et al.*, 1975). The membrane of excitable tissue is thought to have many negatively charged groups on the outer surface i.e. carboxyl and phosphate groups of

membrane phospholipids. Mono- and divalent cations would be attracted to these groups as counterions. The voltage sensors attached to the activation gates were proposed to lie in an electric field compounded from three different sources. Firstly there was the conventional potential difference between the exterior and interior media, secondly there would be the local field from fixed negative charges and lastly the local field from the excess counterions attracted to the surface. Therefore the voltage sensors would be biassed by the local fields which would not be detectable by microelectrodes. This theory is the Gouy-Chapman-Stern theory of ionic double layers used in surface chemistry. The theory was successfully applied to the voltage shifts produced by changes of monovalent, divalent and hydrogen ion concentrations in potassium (Gilbert and Ehrenstein, 1969; Mozhayeva and Naumov, 1970, 1972a, 1972b, 1972c; Brismar, 1973) and sodium channels (Chandler *et al.*, 1965; Hille *et al.*, 1975). Therefore the calcium ions modify firing threshold of excitable cells by acting as counter ions to surface charges.

In their original formulations, Hodgkin and Huxley (1952d) did not consider a possible contribution from calcium ions to the membrane current flowing during an action potential. However in tracer flux studies, Hodgkin and Keynes (1957) found there was an increase in calcium influx during each action potential which amounted to 0.006 pmole/cm^2 in normal sea water and 0.08 pmole/cm^2 in high calcium (112 mM) sea water. The influx of sodium during an action potential was 4 pmole/cm^2 and they concluded that calcium contributed negligibly to the total inward current during excitation.

Baker *et al.* (1970, 1971a, 1971b) reinvestigated this by injecting

the photoprotein aequorin into squid axons. This substance had been found to emit light in the presence of ionized calcium in a quantitative manner (Shimomura *et al.*, 1962; Hastings *et al.*, 1969; Ashley and Ridgway, 1970) and could therefore be used to indicate calcium entry into the axon. Baker *et al.* (1971b) found there were two phases of calcium entry during voltage clamp depolarizations. The early rapid phase showed the same kinetics as the sodium conductance and was sensitive to TTX. They suggested that calcium ions could penetrate sodium channels and contribute to the membrane current during early depolarization. The conductance ratio was estimated as 0.01 gCa/gNa. This entry of calcium was described by the kinetics of gNa i.e. $I_{Ca} = (E_m - E_{Ca})$ 0.01 gNa. The concentration of ionized Ca^{++} in axoplasm was very small and the calculated equilibrium potential was +132 mV. Therefore the very positive E_{Ca} provided a large driving force for inward calcium movement in the voltage range of an action potential.

The second delayed component of calcium entry was strongly dependent on the external concentration of calcium and increased steeply with increasing depolarization up to 80 mV positive to E_m. Thereafter the entry of calcium declined with further depolarization (Baker *et al.*, 1971b). The current declined to a much lower steady state level at any given potential with a time constant on the order of seconds during prolonged depolarization (Baker *et al.*, 1973b). This inactivation was voltage dependent the rate of onset increasing with increasing depolarization. The delayed phase of calcium entry was insensitive to TTX or TEA (Baker *et al.*, 1973a) but was suppressed by magnesium (Baker *et al.*, 1971b) manganese, cobalt, nickel, lanthanum and the drug D-600 (Baker

et al., 1973a, 1973b). Several di- and trivalent cations have all been shown to block calcium entry into various preparations (Hagiwara and Takahashi, 1967; Katz and Miledi, 1969; Miledi, 1971). D-600 is the methoxy derivative of verapamil and both agents block calcium influxes in mammalian heart preparations (Fleckenstein *et al.*, 1969; Kohlhardt *et al.*, 1972). Baker *et al.* (1973a, 1973b) postulated the existence of a late calcium channel in squid axon on these grounds.

This channel seems to be involved in the calcium-dependent regenerative responses observed in squid stellate ganglion by Katz and Miledi (1967, 1969, 1970, 1971). When the sodium spike in the presynaptic nerve terminal was blocked by TTX and the potassium conductance was reduced by TEA, they detected local regenerative responses during depolarizing current pulses. The response was dependent on calcium and because the slope resistance decreased during the plateau of the active response, they concluded that the regenerative response was due to an increase in membrane conductance. Strontium and barium could substitute for calcium to produce regenerative depolarizations, while lanthanum (Miledi, 1971), manganese and to a lesser extent, magnesium were antagonists. Similar results were found in frog spinal ganglion cells (Koketsu *et al.*, 1959; Nishi *et al.*, 1965).

Katz and Miledi (1969) found that the amplitude of the Ca^{++}-spike was always far smaller than expected from estimations of E_{Ca}. One possibility for the discrepancy was that the internal concentration of calcium at the inner surface of the membrane was higher than in the bulk solution. However the reversal potential, from which $[Ca]_i$ can be estimated, closely followed the Nernst equation for calcium suggesting that

this was not the case (Bassingthwaite and Reuter, 1972). Another reason that the spike overshoot was more negative than E_{Ca} was that the peak was determined by an outward current as well as the inward calcium current. Therefore at the peak of the spike, the net current flow would be zero despite the fact that there was still a large driving force for the ion carrying the inward current. The outward current was postulated to be carried by potassium and chloride (Reuter, 1973). Recent evidence from synaptosome preparations suggests that the voltage sensitive channel through which Ca^{++} ions are thought to penetrate during transmitter release possesses a binding site. The site had a pKa of about 6. The inhibitory effect of low pH on transmitter release was postulated to be due to competition between H^{+} and Ca^{++} for this binding site in the channel (Naschshen and Blaustein, 1979).

When the peak potentials of spikes are found to vary logarithmically by about 30 mV for a ten-fold change in the divalent cation concentration such a relationship has been considered good evidence in favour of a selective increase of membrane permeability to divalent cations. However Reuter (1973) pointed out that this relation is only meaningful when the peak of the spike approaches a potential which is determined by the ratio $[Ca]_o/[Ca]_i$, and further the internal calcium concentration should not be appreciably changed during the inflow of calcium ions or during changes in $[Ca]_o$.

Koketsu and Nishi (1969) reported that both sodium and calcium acted as charge carriers during the action potential in frog spinal ganglion cells. Only the sodium component was blocked by TTX. These results indicated that there were different mechanisms for the generation of the

two inward current components. Other workers have reported similar results for the somata of giant neurones in several different molluscs (Kerkut and Gardner, 1967; Chamberlain and Kerkut, 1967; Junge, 1967; Gelduldig and Junge, 1968; Moreton, 1968; Meves, 1968; Krishtal and Magura, 1970; Connor and Stevens, 1971). Most of the neurones continued to be excitable in sodium-free but calcium, strontium or barium containing medium. The slope of the line relating peak potential and the logarithm of the external divalent cation concentration varied between 29.5 mV (Gelduldig and Junge, 1968) and 16 mV (Kerkut and Gardner, 1967; Meves, 1968) for a ten-fold change in external divalent cation. These spikes were not sensitive to TTX. Gelduldig and Junge (1968) found that in calcium-free sodium containing medium, the same neurones exhibited spikes which were TTX sensitive. The membrane behaved like a sodium electrode i.e. about 58 mV change of the peak potential for a ten-fold change in external sodium.

On the basis of voltage clamp experiments, Gelduldig and Gruener (1970) concluded that the major part of the inward current in *Aplysia* neurones is carried by sodium ions. When TTX was added to a sodium and calcium containing medium or if sodium was excluded from the bathing fluid, the maximal inward current was approximately 10% of that obtained in sodium and calcium containing medium. This fraction was dependent on the external calcium concentration and was slower than the sodium sensitive inward current. In squid giant axon (Frankenhaeuser and Hodgkin, 1957) and cockroach giant axon (Narahashi, 1966) the peaks of the sodium spikes were increased by elevating the divalent cation concentration. However these results could be explained on the basis of surface charge

effects and indicated that divalent cations contributed little to the inward current during excitation.

In skeletal muscle, there is a great deal of evidence for a calcium channel in crustacean muscle fibres. Fatt and Katz (1953) observed long lasting action potentials in fibres treated with TEA and the fibres were still excitable when sodium was removed. Fatt and Ginsborg (1958) showed that crayfish muscle fibres were excitable when sodium and magnesium were removed, but not when calcium was excluded from the bathing medium. The amplitude and duration of the action potential was found to increase with increasing external concentrations of calcium. If strontium or barium were the only cations in the solution, no pretreatment with TEA was required. TEA, by blocking the outward potassium flow (Armstrong and Binstock, 1965) allowed the influx of divalent cation to become regenerative. Barium and strontium ions have also been found to reduce the outward potassium current in excitable membranes (Lüttgau, 1954; Cohen *et al.*, 1960; Sperelakis *et al.*, 1967; Standen and Stanfield, 1978). Similar results were obtained in lobster muscle (Werman and Grundfest, 1961) and in barnacle muscle (Hagiwara and Naka, 1964; Hagiwara *et al.*, 1964; 1969; Hagiwara and Takahashi, 1967).

All or none spikes were recorded when the intracellular concentration of calcium was reduced by injection of calcium chelating agents. The overshoot of the spike was sensitive to $[Ca]_o$. Neither sodium nor magnesium could maintain the spikes in the absence of calcium nor influence the spike in the presence of calcium. The relation between log $[Ca]_o$ and peak potential was 29 mV potential change for a tenfold change in external calcium up to 100 mM. Above this concentration, the slope

of the relation was markedly reduced. When Ba or Sr was used to replace calcium, the slope of the peaks of Sr-dependent spikes followed that of a strontium electrode. With Ba, the variation of the peak potential with $\log[Ba]_o$ was much greater than expected for a divalent cation sensitive electrode and was nonlinear.

Hagiwara and colleagues (Hagiwara *et al.*, 1964; Hagiwara and Nakajima, 1966) came to the conclusion that the peak potential was determined by both calcium and potassium conductances. When the free internal concentration of calcium was elevated above 8×10^{-8}M, all-or-none spikes were inhibited. E_{Ca} was more positive than 150 mV under these conditions. The peak potential was well fitted by the ratio log $[Ca]_o/[K]_i$. In voltage clamp experiments, Hagiwara *et al.* (1969) showed that there was an early outward current which varied almost linearly with changes in membrane potential and was time invariant. This component was suggested to be a leakage current carried by potassium ions. The late inward current was sensitive to $[Ca]_o$ and blockade by cobalt. In addition it could be inactivated by conditioning depolarization. They concluded that the inward current was carried by calcium and the two current components used different conductance channels. These experiments were later repeated in a different species of barnacle muscle (Keynes *et al.*, 1973) with similar results. Hagiwara and Takahashi (1967) worked out the potency sequence of various competitive inhibitors of Ca or Sr spikes. The threshold potential was shifted by all these ions to more positive potentials with the same potency. This sequence, La^{+++}, UO_2^{+++} >> Zn^{++}, Co^{++}, Fe^{++} > Mn^{++} > Ni^{++} > Mg^{++}, was similar to that determined for the shift in threshold potential in nerve (Takata

et al., 1966; Blaustein and Goldman, 1968; Hille, 1968b). Hagiwara and Takahashi (1967) however, showed that the inhibition of spikes was independent of the effect on threshold voltage. Tracer flux measurements (Hagiwara and Naka, 1964) and experiments with aequorin (Ashley and Ridgway, 1970) also demonstrated the entry of calcium during depolarizing pulses in barnacle muscle.

In frog muscle, an increase in ^{45}Ca influx during activity was observed by Bianchi and Shanes (1959) and Curtis (1966). The extra influx was about thirty times that found in squid axon amounting to 0.2 pmole/cm^{-2} per impulse in sartorius muscle fibres (Bianchi and Shanes, 1959). This value was slightly higher when estimated in semitendinosus muscle fibres (Curtis, 1966). No electrical activity attributable to calcium was detected originally. However Beaty and Stefani (1976) claimed to have detected a delayed slow spike after blocking the potassium conductance with TEA and removing the chloride in the external solution to negate the chloride shunt. The late activity was sensitive to $[Ca]_o$ and was blocked by cobalt or D-600. It was insensitive to $[Na]_o$ and TTX indicating that it was a calcium spike. As with barnacle muscle, (Hagiwara *et al.*, 1964; Hagiwara and Nakajama, 1966) there was an increase in the potassium conductance of the membrane in addition to the calcium conductance since the peak of the spike did not reach E_{Ca}. The calcium component appeared to be responsible for the delayed repolarization since it inactivated very slowly (Baker *et al.*, 1973b).

This influx of Ca^{++} ions has been postulated to be involved in excitation-contraction coupling by regulating the electrical coupling between the transverse tubular system and the sarcoplasmic reticulum

(Ford and Podolsky, 1970; Chiarandini and Stefani, 1973; Stefani and Chiarandini, 1973; Chandler *et al.*, 1975). Similar observations were made in the slow twitch muscles of the toad (Stefani and Uchitel, 1976). However Miledi *et al.* (1977) found that calcium release from the sarcoplasmic reticulum was unaffected by conditions designed to reduce or abolish entry of external calcium.

Later experiments using voltage clamp demonstrated that the time course of the calcium current was too slow for it to play any part in E-C coupling during a twitch (Stanfield, 1977; Sanchez and Stefani, 1978). However Sanchez and Stefani suggested that this entry of calcium could be important in E-C coupling during long depolarizations.

There is evidence that, in amphibian muscle, there is a late entry of calcium which does not appear to enter by the sodium channel. However exactly what role, if any, it has in excitation-contraction coupling is as yet unclear. There is presently no evidence of such a channel in mammalian skeletal muscle. However regenerative responses which are dependent on calcium have been reported in mammalian and avian muscle cultures. (See: Voltage sensitive ionic channels in tissue cultured skeletal muscle, 2. Transient Channels).

B. Voltage Sensitive Ionic Channels in Tissue Cultured Skeletal Muscle

1. Permanent Channels.

In embryonic or early neonatal muscle, membrane potential was low in the youngest fibres of chick, rat and mouse tested (Boëthius and Knuttsson, 1966, 1970; Hazelwood and Nichols, 1967, 1969; Boëthius, 1969, 1971; Harris and Luff, 1970). As maturation proceeded, the resting membrane

potential rose to adult levels. At the same time, there was a fall in the intracellular sodium concentration whereas the intracellular potassium concentration remained constant in rat muscle (Vernadakis and Woodbury, 1964; Basso *et al.*, 1968; Hazelwood and Nichols, 1968, 1969). Similar observations were made in embryonic and post-hatching chick muscle (Barlow and Manery, 1954; Kano, 1975). Boëthius (1971) suggested that the decrease in intracellular sodium concentration was related to the increase in membrane potential possibly by a change in the active transport of sodium or a decrease in the relative sodium permeability of the membrane.

In primary muscle cultures, the membrane potential was similarly found to develop from initial low levels around -10 mV before and just after fusion, to levels of -50 to -60 mV (Fischbach *et al.*, 1971; Fambrough and Rash, 1971; Dryden *et al.*, 1971, 1974; Powell and Fambrough, 1973; Ritchie and Fambrough, 1975; Spector and Prives, 1977). Thereafter little change in resting membrane potential was found. Higher values between -70 and -95 mV have been reported by other workers (Kano and Shimada, 1971; Kano *et al.*, 1972; Hooisma *et al.*, 1975) although these were less common. Resting membrane potential of explant cultures was maintained at the levels recorded *in vivo* (Li *et al.*, 1959) i.e. about -60 to -70 mV although Harris *et al.* (1973) observed a fall in mean resting membrane potential of -10 mV over a ten day period in culture. Purves and Vrbova (1974) could detect no difference between explants of fast (posterior latissimus dorsi) and slow (anterior latissimus dorsi) muscles of the chick, although differences in E_m levels for fast and slow muscle of neonatal mouse have been reported (Harris and Luff, 1970).

The increase in membrane potential has been correlated with the numbers of nuclei within a fibre (Fambrough and Rash, 1971) fibre thickness (Dryden *et al.*, 1974) and fibre length (Fischbach *et al.*, 1971; Powell and Fambrough, 1973). Resting membrane potential increased as each of the above parameters increased. Hooisma *et al.* (1975) however could detect no rise in resting membrane potential with development and the mean membrane potential of muscle fibres from 2 to 6 days in culture was -71 mV. A similar absence of developmental change in resting membrane potential was observed in the L6 clone of rat muscle fibres (Harris *et al.*, 1971; Kidokoro, 1973, 1975a). Both myoblasts and myotubes had E_m values around -60 mV in the earlier study and -70 mV in the later study by Kidokoro.

The ionic dependence of the resting membrane potential in L6 clone myoblasts and myotubes was studied by Kidokoro (1975a). When external sodium was removed from the medium, the mean resting potential was unaltered whereas it was found to vary linearly with the log of external potassium concentration by 50 mV/decade in both myoblasts and myotubes. From these plots the internal potassium concentration was estimated as 150 mM. The potassium equilibrium potential however was calculated as -83 mV which was more negative than the observed resting potential. Kidokoro ascribed this deviation to leakage of ions from the membrane due to microelectrode penetration. When external chloride was removed, as with sodium the membrane potential was unchanged. Kidokoro (1975a) also investigated the effect of sudden changes in external chloride concentration on continuous recordings of membrane potential (Hodgkin and Horowicz, 1959b). Membrane potential did not change significantly when

the medium was changed from normal to chloride-free saline but the input resistance increased by 10-20% indicating that the membrane was only slightly permeable to chloride.

In primary chick cultures, the internal potassium concentration was about 140 mM whereas the internal sodium concentration fell from 56 mM to 22 mM between 2 day myoblasts and 5 day myotubes (Dryden *et al.*, 1974). The resting membrane potential rose during this period from -10 to -30 mV. Catterall (1975a) estimated that the internal sodium concentration of 5 to 9 day old chick myotubes was about 13 mM based on the uptake of ^{22}Na in the presence and absence of ouabain. In primary rat cultures, the internal concentration of both sodium and potassium remained constant at 13 mM and 153 mM between 3 and 9 days of culture (Ritchie and Fambrough, 1975).

Ritchie and Fambrough (1975) investigated the possibility that the resting membrane potential was determined by electrogenic sodium pumping. Inhibition of electrogenic pumping by a reduction in temperature or by ouabain would have an immediate effect on E_m whereas the inhibition of neutral sodium-potassium exchange would not affect E_m over a short period of time. The membrane potential would gradually fall over a period of hours due to the run down of ionic gradients. When the effect of a short term exposure of rat myotubes to reduced temperature (15°C) or ouabain (up to 10^{-3}M) was studied no effect on the E_m was observed. This therefore would suggest that the membrane potential is determined by the ion distribution according to the passive permeability of the membrane. E_m was found to fall by about 50% after 3½ to 4½ hours incubation with ouabain or incubation at reduced temperature, due to

the gradual accumulation of sodium and loss of potassium.

However electrogenic pumping of sodium did appear to contribute to the recovery of E_m after exposure to low temperatures. E_m had recovered to control levels within 2 to 12 minutes of rewarming to 37°C. This recovery could be partially reversed by reducing the temperature again. If the myotubes were incubated with ouabain, or the metabolic inhibitors DNP and iodoacetate during exposure to low temperatures, then the rapid recovery was prevented. If lithium was substituted for sodium in the incubation medium, then the recovery phase was unaffected. The temperature insensitive portion of the recovery was attributed to the partial re-establishment of ionic gradients. Electrogenic pumping was also detected in chick myotubes after prolonged exposure to acetylcholine. Hyperpolarization of the fibres was observed accompanied by a fall in R_{in} which indicated that membrane permeability was slightly increased. This hyperpolarization was independent of K^+, Cl^-, or Ca^{++} ions and occurred when the external medium contained only NaCl. It did not occur in LiCl or sucrose media and the effect was blocked by DNP, and ouabain. The response was unaffected by atropine and was reversibly blocked by incubation at reduced temperature. Ritchie and Fambrough (1975) suggested that the increased influx of sodium ions during the Ach depolarization activated an electrogenic pump to extrude the excess accumulated sodium ions.

Young myotubes of chick and rat, were found to be relatively insensitive to alterations in external potassium concentration (Fambrough *et al.*, 1974; Dryden *et al.*, 1974; Ritchie and Fambrough, 1975). However as the membrane potential rose, the sensitivity to $[K]_o$ changed until

the membrane potential varied linearly with the logarithm of the external potassium concentration. Fambrough *et al.* (1974) and Ritchie and Fambrough (1975) used a continuously changing sodium and potassium ion gradient. External potassium concentration was measured with a potassium sensitive electrode and lines which were markedly concave were attributed to changing the potassium concentration too rapidly. The linear portion of the curves closely followed the predictions of the constant field equation and the calculated estimates of $[K]_i$ agreed well with the actual measurements of $[K]_i$. Dryden *et al.* (1974) measured E_m at equilibrium after altering the external potassium concentration. The results from both type of experiment were similar and it was suggested that the myotube membrane became more and more selectively permeable to potassium during development. The P_{Na}/P_K ratio fell from 1.00 to 0.43 measured in 2 day chick myoblasts and 5 day myotubes and from 0.4 to 0.07 measured in 3 and 7-11 day rat myotubes (Dryden *et al.*, 1974; Ritchie and Fambrough, 1975).

Ritchie and Fambrough (1975) found that primary rat myotubes were similar to L6 clone myotubes (Kidokoro, 1975a) in that they apparently had a low chloride permeability. Membrane potential did not alter when the bathing fluid was replaced by one containing low chloride in experiments where membrane potential was monitored continuously. If cultures were equilibrated in a high K^+ high Cl^- medium and then exposed to a low Cl^- medium, no transient alteration in membrane potential was found. However repolarization was observed when the high K^+ high Cl^- solution was replaced with standard medium containing 5.3 mM K^+ consistent with the established potassium permeability. Either the myotubes were

extremely permeable to chloride resulting in a very rapid equilibration of $[Cl]_i/[Cl]_o$ with membrane potential or else chloride permeability was low.

In chick muscle cultures, removal of external chloride resulted in depolarization (Dryden *et al.*, 1974) and a decrease in membrane conductance (Dryden and Thomson, 1979). Measurements of internal chloride concentration showed that this fell during myogenesis. E_{Cl} almost exactly equalled the recorded membrane potential. It was concluded that chloride was highly permeable and that it distributed passively according to the membrane potential.

The input resistance of chick muscle fibres in culture fell from relatively high values of 30-40 MΩ to about 1 MΩ or less as the fibres increased in size and E_m rose (Fischbach *et al.*, 1971; Harris *et al.*, 1973). A similar fall in R_{in} was observed in embryonic and post-hatching chicks (Gordon *et al.*, 1977). The fall in input resistance was not apparently caused by changes in the specific membrane resistance (R_m) which remained constant. Fischbach *et al.* (1971) found no correlation between R_m and membrane potential.

When membrane time constants were calculated, differences in the type of analysis applied were apparently more important than species differences. Estimates of R_m calculated from the on or off transients during the passage of a current pulse gave consistently higher values than steady state analysis (see Table 1). In steady state analysis the change in potential at the end of a current pulse of known strength is used to calculate R_{in} by Ohm's Law. Indeed Powell and Fambrough (1973) found that R_m was apparently increased in fibres which were subjected

TABLE 1

PASSIVE MEMBRANE PARAMETERS OF SKELETAL MUSCLE IN TISSUE CULTURE

STEADY STATE ANALYSIS	Membrane Specific Resistance R_m Ωcm^2	Resistance of Myoplasm R_1 Ωcm	Membrane Time Constant τ_m msec	Membrane Capacitance C_m μFcm^{-2}	Length Constant λ μm
Harris *et al.* 1973, Chick explant culture	720	160*	5		
Engelhardt *et al.* 1976, Chick primary culture†	3300	160*	22.3	10.3	1000
Engelhardt *et al.* 1977, Chick primary culture†	3100		25.6	10.7	960
Powell & Fambrough 1973, Mouse primary culture	694 ± 75	109 ± 19	5.4 ± 0.9	8.4 ± 1.2	609 ± 50
Ritchie & Fambrough 1975, 3 day rat primary culture	958 ± 127		3.4 ± 0.4	5.3 ± 1.3	
Ritchie & Fambrough 1975, 9 day rat primary culture	1567 ± 258		5.1 ± 0.4	3.1 ± 0.4	
TRANSIENT ANALYSIS					
Fischbach *et al.* 1971, Chick primary culture	2639 ± 116	220 ± 190	9.95 ± 5.45	3.9 ± 1.45	639 ± 299
Powell & Fambrough 1973, Mouse primary culture	2089 ± 343	132 ± 41	6.9 ± 1.6	4.6 ± 2.0	912 ± 158
Kidokoro 1975a, L6 clone myoblasts	8100 ± 2100	180*	5.7 to 10	1 ± 0.2	
Kidokoro 1975a, L6 clone myotubes	12300 ± 4200		26.5 to 123	4.7 ± 2.1	

* Taken from other authors (Chick: Fedde, 1969; Rat: Albuquerque & Thesleff, 1968)
† 2 to 5 week old cultures

to transient analysis as well as steady state analysis. They considered transient analysis was more liable to error due to complex transient responses resulting from alterations in membrane conductance to potassium ions. These could preclude the determination of any of the membrane constants except R_{in} using the off transient analysis since the constants are derived from the dissection of the off transient into two exponentials of decay. Steady state analysis would be less affected since only τ_m and c_m are derived directly from the on transient. L6 clone myoblasts and myotubes had a significantly higher membrane resistance than the other types of muscle investigated. This is presumably due to the altered ion permeability found in this line i.e. little sodium or chloride permeability.

The 2 to 5 week old leg cultures studied by Engelhardt *et al.* (1976, 1977) had membrane constants which were very similar to those estimated by Fedde (1969) for adult anterior latissimus dorsi (ALD) muscle. This muscle is a slow twitch fibre whereas the posterior latissimus dorsi (PLD) is a fast twitch muscle. The values found in culture appeared to occupy a position intermediate between the values quoted for innervated and denervated muscle (Table 2), suggesting that they could be further modified after the establishment of neural control at innervation. Indeed after denervation R_m, τ_m both increase markedly. C_m also tends to increase but stays relatively constant.

Engelhardt *et al.* (1976, 1977) compared the passive membrane parameters in control chick muscle cultures with cultures to which ventral spinal cord fragments were added 7 days after establishment of the muscle cultures. The passive properties of both sets of cultures were then

TABLE 2

PASSIVE MEMBRANE PARAMETERS OF AVIAN AND MAMMALIAN INNERVATED AND DENERVATED MUSCLE

Reference	Specific Membrane Resistance R_m Ωcm^2	Resistance of Myoplasm R_i Ωcm	Membrane Time Constant τ_m msec	Membrane Capacitance C_m μFcm^{-2}	Length Constant λ μm
Albuquerque & Thesleff, 1968. Rat EDL	559 ± 72	180	1.7 ± 0.1	3.3 ± 0.4	540 ± 50
denervated EDL	759		4.4 ± 1.3	5.8	500
soleus	483 ± 47		1.7 ± 1.0	3.5 ± 0.2	560 ± 50
denervated soleus	458		4.8 ± 1.7	10.8	400
Albuquerque & McIsaac, 1970. Rat EDL	545 ± 79	180	1.5 ± 0.2	2.8 ± 0.5	530 ± 70
denervated EDL	1250 ± 130		6.4 ± 0.8	5.1 ± 1.2	700 ± 100
soleus	512 ± 67		1.5 ± 0.12	2.9 ± 0.5	590 ± 70
denervated soleus	1180 ± 120		5.9 ± 1.00	5.2 ± 1.5	740 ± 30
Westgaard, 1975. Rat soleus	766 ± 24	240 ± 11		3.6 ± 0.07	670 ± 16
denervated soleus	2291 ± 109	301 ± 25		2.7 ± 0.09	717 ± 23
Camerino & Bryant, 1976. Rat EDL	349 ± 29	125		4.2 ± 0.6	
denervated EDL	1371 ± 101			4.8 ± 0.4	

TABLE 2

Continued

Reference	Specific Membrane Resistance R_m Ωcm^2	Resistance of Myoplasm R_i Ωcm	Membrane Time Constant τ_m msec	Membrane Capacitance C_m μFcm^{-2}	Length Constant λ μm
Lorković & Tomanek, 1977. Rat gastroc.	1058	237		4.7	780
denervated gastroc.	1551	128		5.3	940
soleus	843	138		4.7	900
denervated soleus	1801	203		5.3	900
Palade and Barchi, 1977a. Rat diaphragm	445 ± 131	185		3.31 ± 0.12	570 ± 170
Fedde, 1969. Chick ALD	4388 ± 2354	160	35 ± 18	8.2 ± 3.0	1780 ± 510
PLD	561 ± 174		3.7 ± 0.3	7.0 ± 2.1	680 ± 130
Warnick & Albuquerque, 1979. Chick PLD 7 to 8 weeks *ex ovo*	571 ± 70		3.22 ± 0.27	5.88 ± 0.5	622 ± 46
Bryant, 1969. Goat intercostals	1897 ± 86	112 ± 4		4.1 ± 0.2	
Bryant & Camerino, 1976. Goat gastroc.	1052 ± 70	118 ± 4		6.2 ± 0.4	
denervated gastroc.	3221 ± 530			4.8 ± 0.7	

compared 1 to 3 weeks later. Both R_m and τ_m fell by more than 50% from 3300 Ωcm^2 to 1100 Ωcm^2 and 22.3 msec to 8.1 msec (Engelhardt *et al.*, 1976). C_m did not alter significantly but the length constant, λ, fell to 550 μm from 1000 μm. The differences in membrane constants were all attributable to alterations in R_m since from cable theory, $\lambda \propto (R_m a)^{\frac{1}{2}}$ and $\tau_m = R_m C_m$ rather than changes in membrane capacitance. In a second paper, Engelhardt *et al.* (1977) observed a bimodal distribution of R_m in aneural muscle cultures which they attributed to two fibre types, in their thigh muscle cultures. However since Purves and Vrbova (1974) could find no significant differences between cultures derived from fast (PLD) or slow (ALD) embryonic muscle, Engelhardt *et al.* (1977) considered that if their observation was incorrect, then it was probably an artefact due to the presence of embryo extract in the medium. However, after innervation in culture, the proportion of slow-type fibres was significantly reduced and the distribution of R_m measurements more closely resembled that of fast muscle fibres *in vivo* (Fedde, 1969; Gordon *et al.*, 1977). They suggested that a trophic substance from the ventral spinal cord neurones was reducing the specific membrane resistance of the slow-type fibres. They did not consider that functional neuromuscular junction formation was involved since only 5 to 10% of the presumed junctions were functional (Fischbach, 1972).

2. Transient Channels.

Active changes in membrane potential of muscle fibres in culture were first observed by Li *et al.* (1959). Oscillations of the membrane potential of chick explant cultures were found which resulted in the discharge of an action potential or a series of action potentials when the

membrane potential fell below a threshold of about -50 mV. Li (1960) suggested that the origins of the oscillations and fibrillation potentials seen both in denervated muscle and in cultures were metabolic.

Spontaneous action potentials have been seen in other preparations i.e. primary chick and mouse cultures (Fischbach *et al.*, 1971; Kano *et al.*, 1972; Powell and Fambrough, 1973). Action potentials are normally seen after extraneous stimulation. Two types of response have been found, one a typical fast spike with overshoot and the other a much slower depolarization with a plateau phase, often with spikes superimposed (Fischbach *et al.*, 1971; Kano *et al.*, 1972; Land *et al.*, Harris *et al.*, 1973; Kidokoro, 1973, 1975a, 1975b; Spector and Prives, 1977). Fischbach *et al.* (1971) observed that regenerative activity occurred if the resting membrane potential of a fibre was between -55 and -60 mV. Regenerative activity could be detected in young fibres with low E_m if the fibres were firstly hyperpolarized before being depolarized.

The two types of response were then characterized on the basis of their susceptibility to various blocking agents. The spike was abolished by TTX in older cultures whereas in younger cultures TTX resistant spikes were frequently encountered and the slow plateau response was unaltered (Kano *et al.*, 1972; Land *et al.*, 1973; Kidokoro, 1973, 1975b; Kano and Shimada, 1973; Spector and Prives, 1977; Kano and Yamamoto, 1977). Kano *et al.* (1972) suggested that the ionic basis for the two types of activity were different. Land *et al.* (1973) using L6 clone suggested that the fast spike was due to sodium influx and the slow plateau was due to calcium influx or calcium and sodium influx.

The fast component was markedly reduced by the reduction of $[Na]_o$ or TTX whereas the slow component was if anything enhanced. They argued that this could occur if two ionic conductances were responsible and that E_{Na} was less positive than the equilibrium potential for the other ion. Based on the increased response when the external calcium concentration was elevated, they suggested that the other ionic conductance was calcium.

Land *et al.* (1973) found that, contrary to their expectations, the application of manganese or lanthanum greatly prolonged the regenerative response. They suggested that the ions were delaying sodium inactivation or potassium activation. However Kidokoro (1973, 1975b) found that the slow regenerative plateau was eliminated by lanthanum and greatly enhanced if barium was present. The initial fast spike could be initiated in myoblasts but the magnitude of the response was much smaller than that elicited in myotubes. This component was still present if the sodium was replaced by lithium, but no spike was seen in caesium or TRIS medium. Myotubes exhibited a biphasic response which was abolished in sodium free medium. The relation between the peak potential and $\log[Na]_o$ was 32 mV/decade indicating that the membrane was mainly permeable to sodium at the peak of the response, but the second portion of the regenerative potential did not correlate with the external sodium concentration. The second component was enhanced by elevating external calcium and abolished by lanthanum. The initial spike was unaffected by lanthanum. In sodium free-high Ca^{++} or high Ba^{++} medium a slow regenerative response was initiated, which was also blocked by La^{++}.

Similar plateau responses were found in chick muscle cultures

which were susceptible to multivalent cations and unaffected by TTX (Kano and Shimada, 1973; Fukuda *et al.*, 1976a, 1976b). Fukuda (1974) observed a third type of response in chick muscle cultures which produced a long lasting response. This activity was unaffected by removal of Na^+ or by TTX although the initial fast spike was abolished. The second plateau phase was elicited in sodium-free solution and abolished by cobalt leaving the third phase unaffected. The response was all-or-none and afterwards the fibres appeared to be refractory. When the internal chloride concentration was elevated by the passage of a steady hyperpolarizing pulse, the amplitude of the peak potential increased. When acetate was injected, no similar effect occurred. When the external chloride concentration was varied, the peak of the response varied linearly with log $[Cl]_o$ with a slope of 43 mV per tenfold reduction in $[Cl]_o$ - less than the predicted slope of 58 mV. Fukuda *et al.* (1976b) confirmed the presence of the three types of activity in myoballs - muscle fibres which had been induced to round up by treatment with colchicine and were then investigated under voltage clamp (Fukuda, 1975; Fukuda *et al.*, 1976a).

The threshold potential for the generation of the rapid inward current response to step depolarizations was -45 mV. The currents were markedly reduced by removal of Na or addition of TTX and they were concluded to be carried mainly by sodium ions. In sodium free medium, containing elevated calcium, a slow inward current was observed during step depolarizations less negative than -60 mV. The magnitude of the currents was not large enough to establish a quantitative relationship between their magnitude and $[Ca]_o$ but their small amplitude indicated that Ca^{++}

ions did not dominate at the peak of the activity and potassium current was suggested to contribute to the total currents.

When depolarizations of long duration (8 seconds) were investigated, at about -60 mV a small outward current followed by a larger inward current was seen. When the membrane was further depolarized, only inward current was observed. These currents varied with $[Cl]_o$ but were very small or absent if the myosacs were not loaded with chloride prior to clamping. The physiological significance of these slow Cl^- currents is not clear but Fukuda *et al.* (1976a) suggested that chloride ions may play a role during E-C coupling. However the long duration of these currents (seconds to minutes) does not support this idea.

The slow plateau response appeared to be due to both calcium and sodium and the incidence of this component declined with maturity (Kano and Yamamoto, 1977). This paralleled the situation in embryonic and post-hatching chick muscle (Kano, 1975). Kano (1975) proposed that this component of regenerative activity appeared at a certain stage during development and then disappeared in fully differentiated cells. This is supported by the finding of Harris and Marshall (1973) that newborn rat muscle was insensitive to TTX and only developed TTX sensitive action potentials 20 days after birth. The ionic dependence of the action potential was not determined, but the action potential duration of neonate rat muscle was markedly prolonged by barium (Spitzer, 1979).

Similar developmental changes in regenerative activity have been reported in a mouse neuronal cell line (Miyake, 1978). Neuroblastoma cells (N-18) were classified into three stages; an undifferentiated stage, an early developed stage and a fully developed stage. In the

undifferentiated stage, membrane potential was low. When the cells were hyperpolarized to -80 mV, often a small spike was seen at the end of the current pulse. This spike was abolished by 8 mM cobalt but unaffected by removal of external sodium. The second stage was characterized by a higher resting membrane potential about -55 mV and a second component of the active response appeared which was sensitive to micromolar concentrations of TTX but cobalt insensitive. Delayed rectification appeared at this stage also. In the fully developed stage, all-or-none action potentials were found - some consisting only of the fast TTX sensitive component which followed the fast spike. The fast components of both types of response were sensitive to nanomolar concentrations of TTX. The maximum overshoot of the action potential varied linearly with log $[Na]_o$ with a slope of 55 mV/decade. The slow component was unaffected by TTX or removal of Na^+. If potassium conductance was blocked by TEA, and external sodium removed the slow regenerative activity was resolved into an all or none spike which varied linearly with log $[Ca]_o$. Strontium or barium produced similar responses which were susceptible to cobalt. The two components were therefore resolved into a sodium and calcium sensitive response.

During days 4 to 7 *in ovo* embryonic heart beating became progressively more sensitive to inhibition by TTX and progressively less sensitive to inhibition by D600 (Galper and Catterall, 1978). D600 inhibits the plateau phase of the cardiac action potential. This phase is due to an increase in permeability to both sodium and calcium. The heart cells in culture retained this developmental sensitivity to D600 but not to TTX. Because veratridine stimulated influx of $^{22}Na^+$ into cultures

prepared from 3 day or 12 day embryos was inhibited by TTX with a K_i of 1.5 nM, Galper and Catterall (1978) suggested that embryonic heart muscle contained physiologically inactive sodium channels. When cultures were prepared from older embryos much higher concentrations of D600 were required to inhibit beating. There was a 200 fold increase in K_i between cultures prepared from 12 day as opposed to 3 day embryos. When beating recommenced in cultures which had been exposed to TTX, a 10 fold increase in sensitivity to D600 occurred. Galper and Catterall (1978) suggested that as embryonic hearts matured, the slow calcium-sodium channels changed so that they became less sensitive to D600 and reduced their ability to support mechanical activity without the activity of the transient sodium channel which causes the rapid upstroke and overshoot of the action potential in mature ventricle muscle.

The incidence of TTX sensitive spikes increased during development in culture of chick muscle (Kano and Yamamoto, 1977; Spector and Prives, 1977). Spector and Prives (1977) suggested that the lack of correlation of TTX sensitive and insensitive spikes in earlier investigations was due to lack of synchrony. TTX insensitive spikes appear on denervation (Albuquerque and Warnick, 1972; Redfern *et al.*, 1970; Redfern and Thesleff, 1971a; 1971b). The appearance of TTX resistant action potentials can be blocked by inhibition of protein synthesis (Grampp *et al.*, 1972) suggesting that a new population of sodium channels appeared after denervation. Ziskind and Harris (1979) have recently shown that TTX sensitivity of organ cultured adult muscle was restored by reinnervation without activation of the fibres by the nerve. Electrical stimulation alone could not induce the appearance of TTX sensitivity and they

concluded that TTX sensitivity was regulated by a nerve trophic factor. Hasegawa and Kuromi (1978) found that an extract from ventral roots of spinal cord maintained the maximum rate of rise of the action potential at innervated levels and significantly reduced the appearance of TTX insensitivity during organ culture of mouse EDL after denervation. Ach, cyclic AMP, dorsal root and brain extracts could not mimic the effect of the nerve extract.

In chick muscle cultures, (Kano *et al.*, 1979) extract of brain, or spinal cord of chick embryos increased the maximum rate of rise of the action potential (dV/dt_{max}). Extracts prepared from liver were also effective in increasing dV/dt_{max} but extracts prepared from embryonic lung, kidney and muscle were ineffective. The addition of TTX to the medium reduced dV/dt_{max} to about 6 V/sec in all cases. Because the dV/dt_{max} reflects the inward current which flows during a spike, Kano *et al.* (1979) concluded that the nerve extract increased the density of TTX sensitive channels. An increase in the amount of current flowing through a set number of channels was excluded when the driving force for sodium was not altered by treatment of the muscle fibres with nerve extract. Therefore the TTX insensitive channels appear to be an embryonic or less mature stage than the TTX sensitive channels.

The incorporation of transient sodium channels into the membrane was found to be dependent on protein synthesis in cardiac muscle cultures, and their appearance could be induced by messenger RNA (McLean *et al.*, 1976; Nathan and DeHaan, 1978). The appearance of the channels was blocked by cycloheximide. Nathan and DeHaan (1978) demonstrated that after two days in culture, TTX sensitive fast sodium channels were absent

or not functional but after a further 2 to 3 days functional channels appeared. If protein synthesis was inhibited by cycloheximide then they failed to develop. These results indicate that the incorporation of the transient sodium channel into excitable membranes may proceed in a manner similar to that proposed for the Ach receptor complex (see Chapter X).

C. Rationale

From the introduction, it is evident that although there is a vast literature dealing with the transient channels of excitable cells, very little is known about the resting membrane. Excitable cells maintain a high potential difference across the membrane in contrast to non-excitable cells. The mechanism by which this elevated E_m is acquired is incompletely understood, but it is known to be essential for the maintenance of excitable properties (Hodgkin and Huxley, 1952c).

The progression from non-excitable to excitable membrane can be followed during the differentiation of skeletal muscle *in vitro*. Based on the observation that the P_{Na}/P_K ratio fell, Dryden *et al.* (1974) proposed that the membrane permeability to potassium rose to account for the rise in resting membrane potential. The present investigation set out to test this hypothesis by firstly determining the resting membrane conductance of chick pectoral muscle on a daily basis during the period of rapid differentiation. Secondly the individual contributions from the major ions to resting membrane conductance were determined and compared with the predictions from the hypothesis. Thirdly the effects of various pharmacological agents, held to be inhibitors of ion conductances were examined throughout differentiation.

II. MATERIALS AND METHODS

A. Tissue Culture

1. Tissue Culture Medium.

The solutions used during preparation of the tissue were Earle's Balanced Salt Solution (BSS) (Earle, 1943) and the growth medium. This medium (termed whole MEM) was Eagle's Minimum Essential Medium (MEM) (Eagle, 1959) containing 5% of a 50% v/v whole embryo extract and 15% horse serum. The whole MEM always contained penicillin G (100 iu/ml) streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml).

Whole embryo extract was prepared by pressing 10-11 day chick embryos through a 50 ml syringe. To this extract, an equal volume of BSS was added and the whole was deep frozen until required. Before use, the extract was allowed to thaw for 24 hours prior to centrifugation at an average force of 20,700 g for 3 hours. The supernatant was then sterilized by filtration through a Millipore filter (YY 30 142 30, Millipore Corp. Bedford, Massachussetts, USA). For preparation of 0.05% trypsin solutions (Difco Labs. Inc. Detroit, Michigan, USA) a calcium and magnesium free BSS (Table 3) was used.

Rat tail collagen was prepared by the method of Ehrmann and Gey (1956). A tail was steeped in 70% ethanol and the skin stripped off. The tendons were dissected free and placed in 0.1% sterile acetic acid solution for 24-48 hours at 0-4°C. The residue was then centrifuged at an average force of 20,700 g for 3 hours. The supernatant was diluted further with 0.1% acetic acid solution if needed and then refrigerated until required.

TABLE 3

COMPOSITION OF SOLUTIONS FOR TISSUE CULTURE

Constituent (mg/litre)	Eagle's MEM	Earle's BSS	Ca^{++}-Mg^{++} Free BSS
NaCl	6800.0	6800.0	7005.0
KCl	400.0	400.0	400.0
$CaCl_2 \cdot 2H_2O$	200.0	265.0	
$MgSO_4 \cdot 7H_2O$	200.0	200.0	
NaH_2PO_4	140.0	140.0	54.0
Na_2HPO_4			110.0
$NaHCO_3$	2200.0	2200.0	
Na phenol red	10.0	10.0	10.0
Glucose	1000.0	1000.0	1000.0
L-arginine	105.0		
L-cystine	24.0		
L-glutamine	292.0		
L-histidine	31.0		
L-isoleucine	52.5		
L-leucine	52.4		
L-lysine	58.0		
L-methionine	15.0		
L-phenylalanine	32.0		
L-threonine	48.0		
L-tryptophan	10.0		
L-tyrosine	36.0		
L-valine	46.0		
D-calcium pantothenate	1.0		
Choline chloride	1.0		
Folic acid	1.0		
i-Inositol	2.0		
Nicotinamide	1.0		
Pyridoxal HCl	1.0		
Riboflavine	0.1		
Thiamine HCl	1.0		

2. Tissue Sources.

All cultures were prepared from chick embryos of either the White Leghorn or Hubbard strain. Both were obtained from the University of Alberta Farm, Edmonton, Alberta. No differences were observed between strains and data from both are presented although the majority of experiments were performed using White Leghorn embryos.

3. Preparation of Cultures.

For leg muscle cultures, single cell suspensions of myoblasts were obtained by the method of Konigsberg *et al.* (1960). Embryos of 10 days incubation age were used. After removal of the embryo from the egg and extra-embryonic membranes, the skin was peeled away from both legs and the legs severed from the body at the ischial crests. The muscle tissue was dissected free from the bone and minced with a fine pair of scissors. The minced tissue was placed in 10 ml of 0.05% trypsin solution for 30 minutes at 37°C. The digestion process was halted by the addition of 10 ml of whole MEM. The horse serum contains trypsin inactivating factors. Final dissociation of the cell aggregates was achieved by aspiration of the solution in a Pasteur pipette for 1-2 minutes. The cell suspension was filtered through several layers of gauze to remove cell aggregates and the isolated cells were then spun down in a bench top centrifuge at low speed for 5 minutes. The supernatant was discarded and the muscle cells were separated from the heavier red blood corpuscles by use of a fine Pasteur pipette. The cells were resuspended in 10 ml Whole MEM and the number of cells was counted using a haemocytometer.

For breast muscle cultures, embryos of 11 days incubation age were

used. The pectoral muscle was dissected free and washed with BSS. The BSS was then removed by aspiration and the tissue minced with fine scissors. The minced tissue was then placed in 10 ml of whole MEM and dissociation was achieved by repeated aspirations in a Pasteur pipette for 10 minutes. The cell suspension was filtered through gauze and thereafter prepared in the same manner as outlined previously for leg muscle.

Cultures were grown in 30 mm plastic petri dishes (Nunc, Kamotrup, Denmark) which were coated with collagen (Hauschka and Konigsberg, 1966). Cells were plated at 1×10^5 cells/ml, 2 ml of cell suspension being added to each plate. Cultures were incubated at 37°C in a water saturated atmosphere of 5% CO_2 in air. The growth medium was normally changed twice a week. Some leg cultures were treated with medium containing 10^{-5}M 1-β-D-arabinofuranosyl cytosine (AraC) (Sigma, St. Louis Missouri, USA) for either 36 or 72 hours after the onset of rapid myoblast fusion. Enough whole MEM was prepared on each occasion so that all the plates received the same medium. Half of the plates received ara-C and when the drug was removed, the medium was replaced in the whole culture. Thereafter medium was changed twice a week.

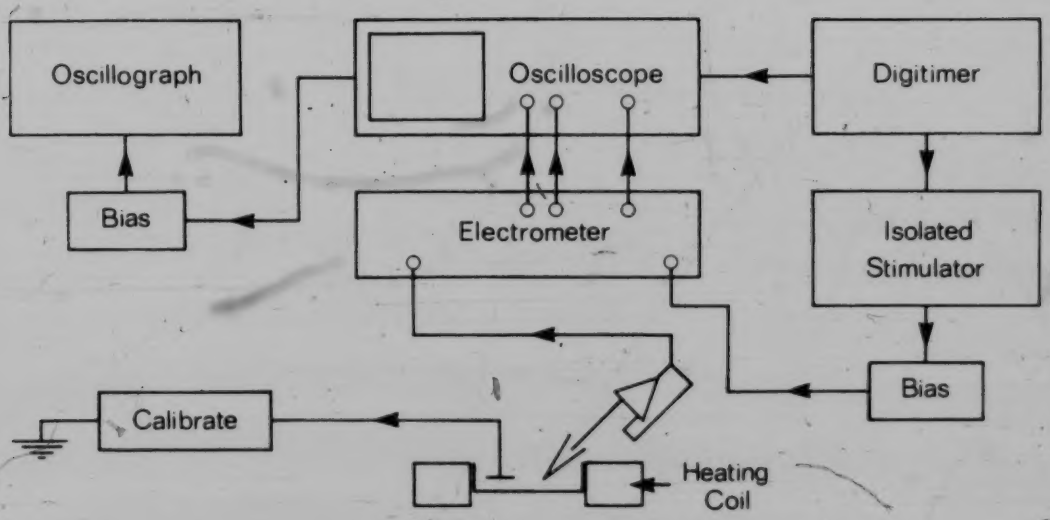
B. Electrophysiological Recording and Drug Application

1. Experimental Equipment.

A schematic diagram of the equipment is shown in Figure 1. The cultures were observed with a phase contrast inverted microscope (Reichert, "Biovert"). Medium was continuously changed in the culture dish by a flow through system supplying bicarbonate buffered BSS. The rate of flow was adjusted to 1 ml/minute except when a solution change was in

Fig. 1. Experimental apparatus.

Signals from the recording microelectrode were fed through a WPI M701 microprobe electrometer (W.P. Instruments, Hamden, Conn. USA) to a Tektronix R5031 dual beam oscilloscope (Tektronix Inc., Beaverton, Oregon. USA). Command pulses from the Digitimer, D4030 (Digitimer Ltd., Welwyn Garden City, Herts., England) were fed to the oscilloscope and to the isolated stimulator, type 2533 (Digitimer Ltd., Welwyn Garden City, Herts., England). Current pulses from the stimulator could then be passed to the recording electrode via the electrometer. If required an offset voltage could be applied to hyperpolarize fibres impaled by the electrode. Calibration pulses were applied by means of a CRO Calibrate Unit, 3140 (Digitimer Ltd., Welwyn Garden City, Herts., England). Permanent recordings of the voltage trace were made on a George Washington Oscillograph, 400 MD/2 (Washington Palmer, Sheerness, Kent, England).


Oscillograph
Oscilloscope
Digitimer
Bias
Electrometer
Isolated
Stimulator
Bias
Calibrate
Heating
Coil

progress. Then the flow rate was increased to 10 ml/minute for the first minute of perfusion. Humidified CO_2 enriched air was blown over the surface of the BSS which was warmed by a heating circuit (Bonkowski and Runion, 1976). The circuit also maintained the temperature of the culture at 37°C during experiments.

Microelectrodes for intracellular recording were of glass, 1.2 mm external diameter, (no. 7740 Corning Glass New York, N.Y. USA) pulled on a horizontal microelectrode puller (Model M1 Industrial Science Associates Inc. Ridgewood N.Y. USA). Bevelling was performed on a K.T. Brown type microelectrode beveller (Model BV-10 Sutter Instrument Co. San Francisco Ca. USA). The electrodes were filled to the shank with distilled water by use of a 150 W. incandescent tungsten lamp. Subsequently the barrel of the electrode was filled with 3 M KCl or 2 M potassium acetate delivered via a needle. The bulk solution was then changed from distilled water to either 3 M KCl or 2 M potassium acetate and the tips of the electrodes were allowed to fill with the salt solution by passive diffusion overnight. Occasionally capillary glass microelectrodes (GC 120F-10, Clark Electromedical Instruments, Pangbourne, Reading, England; or 11 000 07, Hilgenberg Glas, Malsfeld, W. Germany) were used. Electrodes with resistances of 20-40 MΩ were used, or if bevelled, electrodes of final resistance 10-20 MΩ were used. Electrodes for bevelling were of higher initial resistance (30-60 MΩ).

Recording electrodes were mounted on a Leitz micromanipulator. The potential differences between the recording microelectrodes and a chlorided silver wire bath electrode were monitored via a WPI. M701 electrometer on an oscilloscope (Tektronix R5301) and permanently

recorded on a George Washington 400 MC/2 Oscillograph (Washington-Palmer, Sheerness, England). Square wave pulses of current supplied by an isolated stimulator (Type 2533, Digitimer Ltd., Welwyn Garden City, London, England) were passed through the recording electrode using the bridge circuit of the M701 electrometer. The bridge balance was continually monitored before and after penetration of a fibre. Measurements of input resistance (R_{in}) after which the bridge was found to be unbalanced were discarded. For estimates of R_{in} the duration of the stimulus current was set at 100 msec and the amplitude was varied between 0 and 2.5 nA. The XY mode of the oscilloscope was used to display the resulting current-voltage relationship directly. R_{in} was measured in both the hyperpolarizing and depolarizing directions. The slope of the line was measured over the current range of 0-1.25 nA and taken to be R_{in} for the individual fibres. In experiments where regenerative activity was investigated, both the amplitude and duration of the stimulus current were varied until threshold was reached.

2. Measurement of Membrane Potentials and Input Resistance.

Intracellular membrane potentials (E_m) and R_{in} were measured using the equipment described above. For each culture a series of control measurements of E_m and R_{in} were made in identified fibres in BSS. The flow through system was then switched to a reservoir containing the test solution. This was allowed to perfuse for 15 minutes before the measurements were repeated in the same fibres. If a second test solution was used, then this was also allowed to perfuse for 15 minutes before a third series of measurements were taken. In some experiments it was found necessary to buffer the BSS with 18 mM sodium HEPES (N-2-hydroxyethylpipera-

zine-N'-ethanesulphonic acid) (Good *et al.*, 1966) in preference to the standard bicarbonate buffer. Manganese and cobalt were found to be incompatible with the bicarbonate buffer in BSS. The use of HEPES to buffer the control solutions instead of bicarbonate had no effect on estimates of E_m or G_m.

3. Microperfusion.

Micropipettes were prepared by pulling capillary glass as in preparing microelectrodes and then breaking back the tips to between 5 and 10 microns in diameter under a microscope with a micrometer set in the eyepiece. The micropipettes were filled by suction with either the control (BSS) or test solution. A stock solution of condylactis toxin (CTX) (1 mg/ml) in BSS was prepared and kept frozen at -20°C until required. The toxin solution was used at a concentration of 0.2 mg/ml in BSS. The connecting tubing to the syringe was filled with silicone fluid (10 cSt D.C. 200, Dow Corning, Calgary, Alberta, Canada). The syringe was inserted in a perfusion pump (Arnold Microapplicator Type L.V. 65; Burkard Manufacturing Co. Ltd. Rickmansworth, Herts, England) which was set to deliver 0.5 µlitre per cycle. Fibres were penetrated with 3 M KCl electrodes and a steady D.C. voltage was supplied to maintain the fibres at -80 mV. Constant current pulses of 100 msec duration were passed through the recording electrode and the resulting shift in potential recorded on the pen recorder. The micropipette was then brought into close proximity to the fibre under investigation and 10 µl of solution were delivered per fibre. Under these conditions the fibre is bathed with a known concentration of toxin for a period of 1-2 minutes before diffusion reduces the concentration at the fibre surface.

C. Drugs and Materials Used

The solutions used during the preparation of the leg and breast muscle for culture were Earle's BSS, Eagle's MEM and calcium and magnesium-free BSS. Their composition is given in Table 3. All solutions were sterilized by filtration. The tissue culture components were obtained from either Gibco, Calgary, Alberta, Canada, or Flow Laboratories, Inglewood, Ca., USA.

The bathing media and ion replacement solutions used in the experiments are detailed in Table 4. The sodium-free solution (Solution D) was buffered with half the normal concentration of potassium HEPES (Good *et al.*, 1966) to maintain the potassium concentration similar to that found in BSS (Solution A). The pH of this medium was checked (pH 7.0) and found to be similar to that of BSS (pH 6.8-7.0).

The chemicals used to prepare the solutions in Tables 3 and 4 were of reagent grade. Other chemicals and drugs used were: 4-aminopyridine (4-AP); anthracene-9-carboxylic acid (9-AC); 3,4-diaminopyridine (3,4-DAP); veratridine (all from Aldrich Chemical Co. Inc., Milwaukee, Wis. USA.); condylactis toxin (CTX); d-tubocurarine (d-TC); N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid (HEPES); piperazine-N,N'-bis-[2-ethanesulphonic acid] (PIPES) (all from Sigma St. Louis, Mo. USA.); cobalt chloride; manganese chloride (both from Fisher Scientific Co., Fair Lawn, N.J. USA.); tetraethylammonium chloride (TEA) (Eastman Kodak. Co. Rochester, N.Y. USA.); tetrodotoxin (TTX) (Sankyo Co. Ltd., Tokyo, Japan); verapamil (a gift from A.G. Knoll Labs, Ludwigshafen, FDR.).

TABLE 4

COMPOSITION OF BATHING SOLUTIONS FOR ELECTROPHYSIOLOGY

Constituent mM	A	B	C	D	E	F	G	H
NaCl	115.7	115.7	15.7			115.7		118.6
KCl	5.4		5.4			5.4		5.4
$CaCl_2 \cdot 2H_2O$	1.8	1.8	1.8	1.8		1.8		1.8
Glucose	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
$MgSO_4 \cdot 7H_2O$	0.8	0.8	0.8	0.8	0.8	0.8	0.8	
NaH_2PO_4	1.0	1.0	1.0		1.0			
$NaHCO_3$	26.2	26.2	26.2		26.2			
Na phenol red	0.03	0.03	0.03		0.03	0.03	0.03	0.03
CsCl		5.4						
TEA Cl			100.0					
Choline Cl				115.7				
KH_2PO_4				1.0				
HEPES				9.0				18.0
KOH				4.5				
Sucrose							60.0	
Ca acetate					1.8		1.8	
Na isethionate					114.4			
K CH_3SO_4					5.4			
$ZnCl_2$						0.5	0.5	
PIPES						5.0	5.0	
Na_2SO_4							60.0	
K_2SO_4							2.7	
NaOH						5.0	5.0	9.0
$LaCl_3$								1.8

A BSS
B K^+-free BSS
C Low Na^+ BSS for TEA^+
D Na^+-free BSS
E Cl^--free BSS
F Zn BSS
G Cl^--free BSS for Zn
H La BSS

D. Calculation of Membrane Parameters and Statistics

1. Membrane Parameters.

R_{in} was determined from the steady state change in potential resulting from the passage of a known current through the recording electrode as described in section B.1 using the 'square pulse analysis' of Fatt and Katz (1951). The diameter of each myotube was determined using a calibrated micrometer scale set in the eyepiece of the microscope. A correction value of 0.8 was applied to each measurement of fibre diameter because chick myotubes are irregularly elliptical rather than circular in cross-section. This value was determined from measurements of fibre circumference in electron micrographs of transverse sections (Figure 2). The time taken for the voltage change in response to the passage of current to reach 83% of the maximum was taken as an estimate of the membrane time constant, τ_m. This value was calculated using the infinite cable model of Hodgkin and Rushton (1946) as modified for the short cable considerations (Stefani and Steinbach, 1969). The relationship V_o/I_o was taken as a measure of R_{in} where V_o was the potential at $x = 0$ in volts and I_o was the current injected at $x = 0$ in amps. x was the distance between current injecting and potential recording microelectrodes measured in centimetres (Schanne, 1969). In this case $x = 0$ because one electrode was used both to record and inject current.

The specific membrane resistance was calculated from the relation:

$$R_m = \frac{\pi^2 d^3 (R_{in})^2}{R_i}$$

d = fibre diameter in cm.
R_{in} = input resistance in Ω.
R_i = specific resistance of the cytoplasm in Ωcm.

R_i was taken as 160 Ωcm (Fedde, 1969). Specific membrane conductance G_m was taken as the reciprocal of R_m.

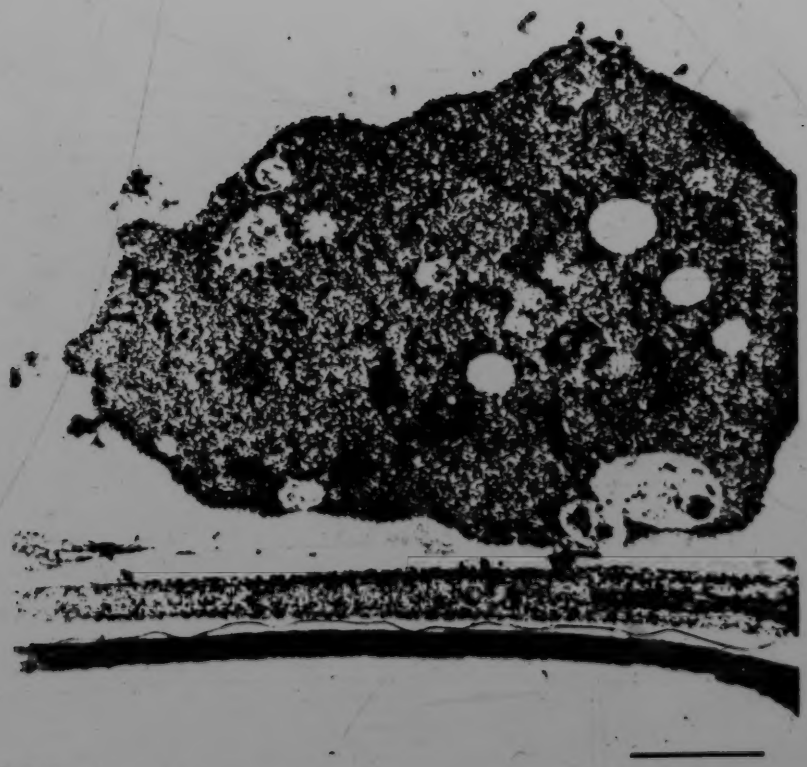


Fig. 2. Electron micrographic transverse section of a chick skeletal muscle fibre in culture. The micrograph was kindly prepared by Dr. A.F.R. Wolfe by the method of Brinkley *et al.*, 1967. The calibration bar equals 1 μm.

Fibres which were long, relatively unbranched and of fairly uniform diameter were chosen for estimations of input resistance. The voltage-current relationship was required to be linear for a 20 mV hyperpolarization or depolarization. Fibres which exhibited rectification over this range were discarded. Fibres were generally 1-2 cm in length and estimates of the length constant of chick muscle in culture vary from 600-1000 μm (see Table 1). The ratio of fibre length to that of length constant is required to be > 4.5 to comply with short cable considerations. Fibre branching in cultures of chick muscle renders estimates of fibre length meaningless and so this requirement was not strictly met by many of the fibres under investigation despite efforts to use unbranched lengths of fibre. However the fibres are less well approximated by the spherical cell model for the calculation of specific membrane resistance. Although the absolute individual values calculated for R_m may be in some error, the relative changes in R_m produced by the test solutions employed will remain constant.

2. Statistics.

Since measurements of R_{in} were made in the same fibres before and after the application of the test solution Student's paired or unpaired *t*-test was used to analyze the results. $P < 0.05$ was set as the limit of significance throughout. Values of $P > 0.05$ are therefore non-significant. In the experiments using ara-C, the relationship between age of fibres and action potential parameters was analyzed. A straight line was fitted to the data using regression analysis, and the coefficient of correlation (r) determined. When the dimensions of the symbol representing the data point exceeded those of the standard error, no standard error bar was displayed in subsequent figures.

III. EFFECTS OF 1-β-D-ARABINOFURANOSYL CYTOSINE ON THE ELECTROPHYSIOLOGICAL DEVELOPMENT OF CHICK SKELETAL MUSCLE IN CULTURE

A. Introduction

One of the advantages promised by the technique of tissue culture is that relatively homogeneous populations of cells can be obtained for experimental purposes. While this may not be suitable in neurone cultures in view of the considerable supportive and maturational role of glial cells, in muscle cultures, the removal of non-myogenic tissue from primary cultures appeared desirable for a number of investigations. As the cultures mature, overgrowth of the myotubes by other cell types (mainly fibroblasts) renders penetration of the myotubes for electrophysiological recording difficult.

1-β-D-arabinofuranosyl cytosine (ara-C), an inhibitor of DNA synthesis originally reported to have no effect on RNA or protein synthesis (Silagi, 1965; Cohen and Studzinski, 1967; Graham and Whitmore, 1970) has been employed in muscle culture to remove unwanted replicating cells from primary monolayer cultures of differentiated fusing myoblasts (Fischbach, 1972; Dryden *et al.*, 1974). Early work demonstrated that there was no morphological alterations, no difference in chemosensitivity and no apparent effect on resting membrane potential (Fischbach, 1972; Fischbach and Cohen, 1973; Dryden *et al.*, 1974). More recently, Ritchie and Fambrough (1975) noted that cultures treated with ara-C yielded thinner myotubes and such cultures seemed to be more fragile as judged by the higher frequency of unsuccessful impalements.

Since the purpose of this series of investigations was to determine the development of ion channels in the normal myotube membrane

during differentiation, a preliminary study of the effects of ara-C on the normal electrophysiological maturation process was carried out.

B. Results

1. Effects on Morphology of Myotubes.

The morphological development of myotubes treated with 10^{-5}M ara-C has been described previously (Fischbach, 1972). As found then, no difference was observed between treated and control cultures in the acquisition of striations, orientation of nuclei or branching of the myotubes. In long term cultures, fibroblast overgrowth of the myotubes makes successful impalement more difficult. Treatment with ara-C however does not prevent this problem but only delays the onset of overgrowth. Therefore after 8-9 days in culture, measurements could only be accepted if a satisfactory value for resting membrane potential was obtained, together with the appearance of a membrane time constant on the passage of current to confirm that a penetration was successful.

2. Effects on Membrane Potential and Fibre Diameter.

Figure 3 shows the development of resting membrane potential (E_m) during maturation in culture over a twelve day period. Membrane potentials in the ara-C treated myotubes consistently increased more rapidly than in control cultures. The 36 hr treated myotubes attained a significantly higher value than the 72 hr treated myotubes from the 6th to the 8th days of culture (days 6 and 8, $p < 0.01$; day 7, $p < 0.05$). In control cultures, the resting membrane potential rose more slowly than in treated cultures. Although the mean final values attained (about -55 mV) were not significantly different from the highest values noted in

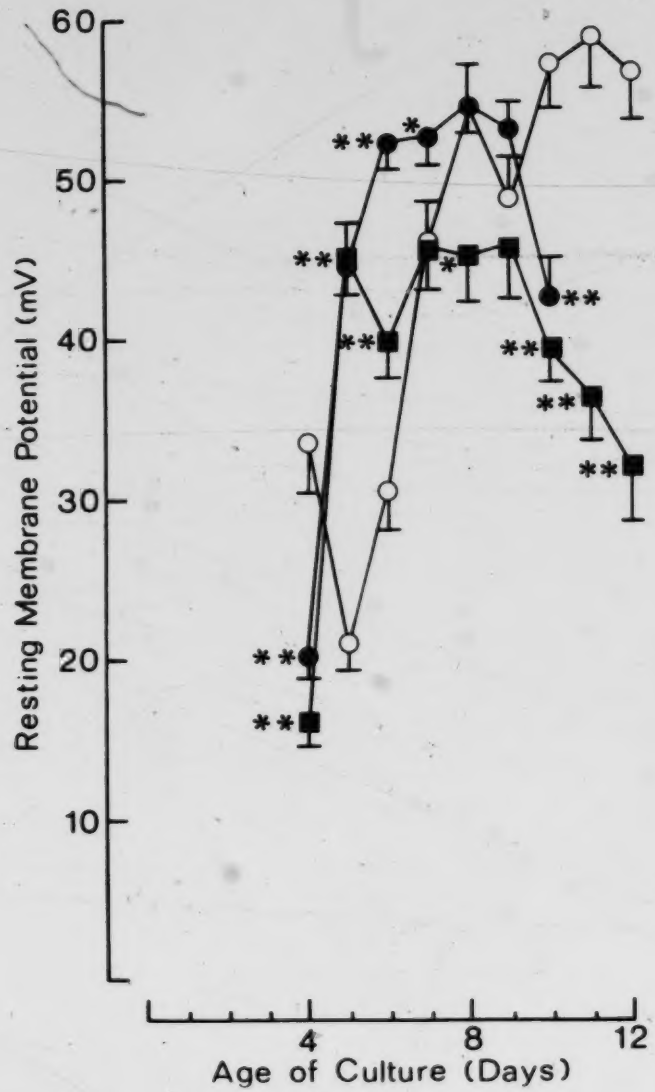


Fig. 3. Effect of 10^{-5}M ara-C on developing myotube membrane potential. (○) control; cultures exposed to ara-C for (●) 36 hr; (■) 72 hr. Each point is the mean ± SEM of at least 21 observations from a single culture dish. Cultures measured on successive days were established at the same time. Treated cultures were compared to the control using Student's unpaired *t*-test. * $P < 0.05$, ** $P < 0.01$.

36 hr treated cultures, they were nevertheless maintained, unlike the treated cultures, where the mean resting membrane potential fell progressively after the 9th day of culture (days 10-12, $p < 0.01$).

The mean fibre diameter of these same myotubes is illustrated in Figure 4. Initially there is no significant difference in diameter between control cultures and those exposed to ara-C. However, myotubes exposed to ara-C for 36 hr were significantly thinner than controls after 7 days in culture ($p < 0.01$). After 9 days, the diameters of the ara-C treated myotubes were significantly less than in controls (36 hr exposure, $p < 0.01$; 72 hr exposure, $p < 0.05$). On the tenth day of culture, cells exposed to ara-C for 72 hours remained significantly smaller in diameter than controls ($p < 0.01$) although cultures exposed for 36 hours reverted back to control levels.

The failure of ara-C treated myotubes to develop diameters comparable to control myotubes agrees well with a similar observation made by Ritchie and Fambrough (1975). Control diameters fell after ten days of culture and were not significantly different from those of 72 hours ara-C treated myotubes. The fall in fibre diameter seen in both control and ara-C treated cultures is attributed to the fact that the criteria for acceptance of a measurement were most often satisfied by myotubes at the periphery of the culture which are thinner than those in the centre where fibroblast overgrowth made penetration and diameter measurement unreliable. However, the reduced diameter of the ara-C treated fibres is always over and above the apparent reduction in diameter imposed by sampling.

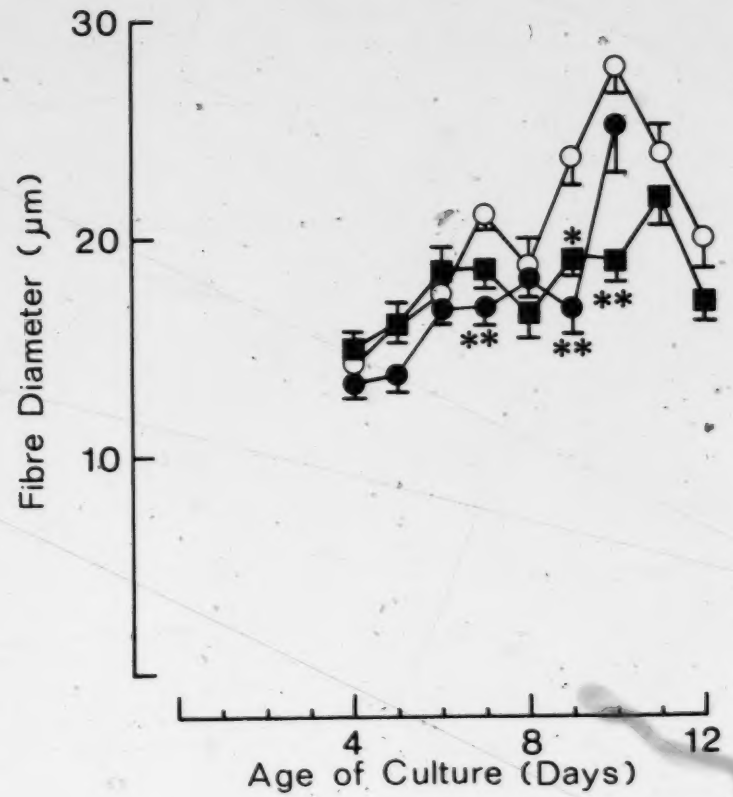


Fig. 4. Effect of 10^{-5}M ara-C on diameter of fibres used in Fig. 3. (○) control; cultures exposed to ara-C for (●) 36 hr; (■) 72 hr. Each point is the mean ± SEM of at least 20 observations. Treated cultures were compared to the control using Student's unpaired *t*-test. * $P < 0.05$, ** $P < 0.01$.

3. Effects on Membrane Time Constant and Resistance.

No significant difference in mean R_{in} and R_m was detected between control and ara-C treated preparations despite the fact that ara-C retarded the increase in the diameter of myotubes. This was attributed to the large variation in resistance measurements observed between individual fibres, although it was noted that the variance of measurements in treated cultures was in the main considerably greater than that of controls.

However, when membrane time constants (τ_m) were calculated for the control and cytosine-treated preparations, significant differences were noted (Table 5). τ_m for the 72 hour treated cultures was twice that for the control ($p < 0.001$) whereas τ_m for the 36 hour treated cultures was intermediate between the two. This value was closer to that of the control τ_m but still significantly different from it ($p < 0.02$). The values for the two ara-C treatments are also significantly different ($p < 0.005$).

4. Effects on Active Membrane Parameters.

The development of electrical excitability in both control and ara-C treated cells was also investigated. Young myotubes in 2-3 day cultures exhibited passive, symmetrical responses to equal pulses of hyperpolarizing and depolarizing current. Myotubes in older cultures with an E_m of less than -50 mV produced similar passive responses to the passage of current. The onset of regenerative activity was first noted in four day control cultures but never in the ara-C treated cultures until the fifth day after plating. There was no obvious correlation between fibre diameter or input resistance and the appearance of this regenerative activity.

TABLE 5

COMPARISON OF τ_m MEASUREMENTS OF NORMAL AND ARA-C TREATED MYOTUBES

Treatment	τ_m (msec) $\bar{x}$	SEM	n
Control	19.9	1.4	33
36 hr ara-C	25.8	2.2	16
72 hr ara-C	36.2	2.3	23

In both the control and 36 hr ara-C treated cultures, the development of the action potential can be followed from the appearance of slow regenerative potentials to fast spikes with overshoot (Figure 5 a-f). The fast spikes are defined as those regenerative potentials with a maximum rate of rise (dV/dt_{max}) of at least 10 V/sec and are associated with twitches of myotubes. The slow regenerative potentials have dV/dt_{max} values in the range 0.1 to 10 V/sec and are associated with sustained contracture (Spector and Prives, 1977).

In all cultures, the fast spikes were abolished by 1×10^{-7}M tetrodotoxin (TTX), a specific blocker of the fast sodium channel (Narahashi, 1974); the slow regenerative potentials were abolished by 1.8 mM $LaCl_3$. TTX did not affect the slow regenerative potentials and often in younger myotubes, a slow potential was revealed in the presence of the toxin which was subsequently abolished by the $LaCl_3$ (Figure 6).

In myotubes which were exposed to ara-C for 72 hr, this sequence of development was not so predictable (Figure 5 g-j). A great variation was found in the type of potential produced in response to the passage of current. Although more rapid regenerative potentials were found in older cultures which had been exposed to ara-C for 72 hours (10 days and upwards), the amplitude of the regenerative potential rarely exceeded 28 mV (Figure 7C) and dV/dt_{max} never exceeded 11 V/sec (Figure 8C). However, in control cultures the amplitude of the spike and dV/dt_{max} increased as the fibres matured (Figures 7A and 8A) with a high degree of correlation ($P < 0.001$). In myotubes exposed to ara-C for 72 hours, the correlation of both these parameters with age is very poor (Figure 7C, NS; Figure 8C, $0.05 < P < 0.1$). When the duration of exposure to

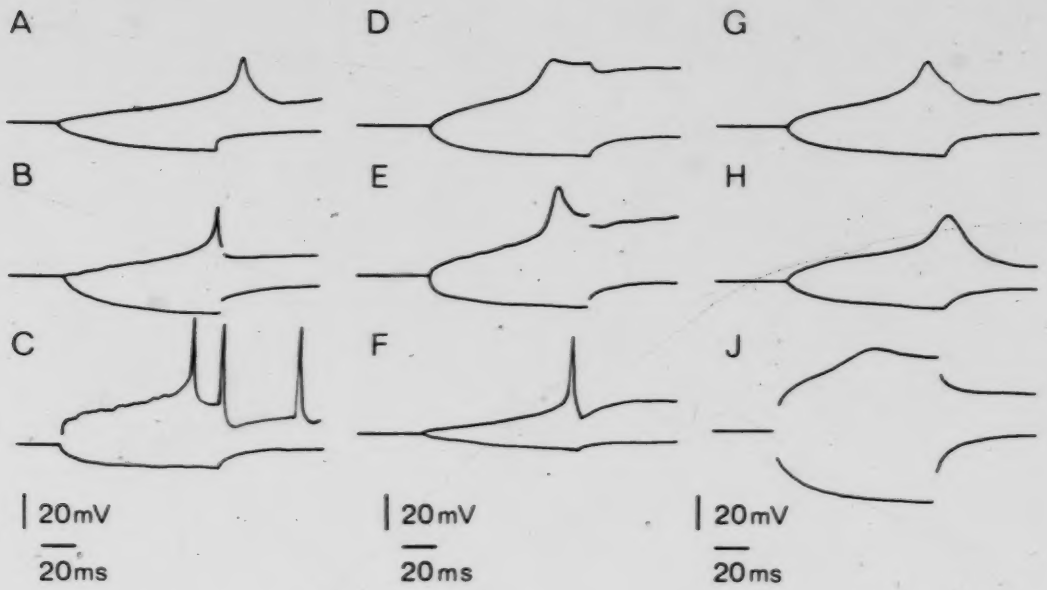


Fig. 5. Development of regenerative electrical activity in myotubes at 3 stages of maturation. (a-c) control, 5, 6, and 8 days after plating respectively. (d-f) 36 hr 10^{-5}M ara-C treated culture, 5, 6, and 8 days after plating respectively. (g-j) 72 hr 10^{-5}M ara-C treated culture, 5, 6, and 8 days after plating respectively. Current pulses of 100 msec duration applied at the following strengths: a. 3.75 nA, E_m = 82 mV; b. 8.75 nA, E_m = 82 mV; c. 6.25 nA, E_m = 66 mV; d. 2.1 nA, E_m = 73 mV; e. 0.75 nA, E_m = 86 mV; f. 1.5 nA, E_m = 72 mV; g. 2.5 nA, E_m = 66 mV; h. 1.6 nA, E_m = 72 mV; j. 3.6 nA, E_m = 76 mV.

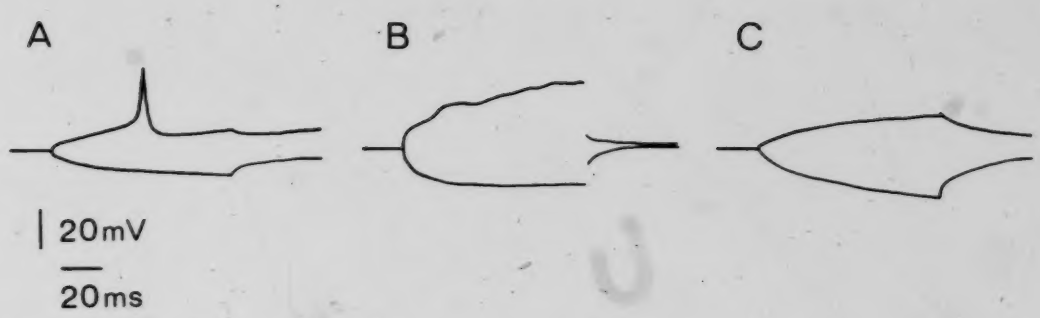


Fig. 6. Single sample fibre from 36 hr 10^{-5}M ara-C treated fibre 6 days after plating showing pharmacological resolution of regenerative potentials. a. E_m = 76 mV current strength 3.5 nA; recording in BSS. b. E_m = 87 mV current strength 5.5 nA; recording in BSS containing 3 x 10^{-7}M TTX. c. E_m = 63 mV current strength 4.75 nA; recording in modified BSS containing 1.8 mM $LaCl_3$ 10 minutes after changing from solution in b.

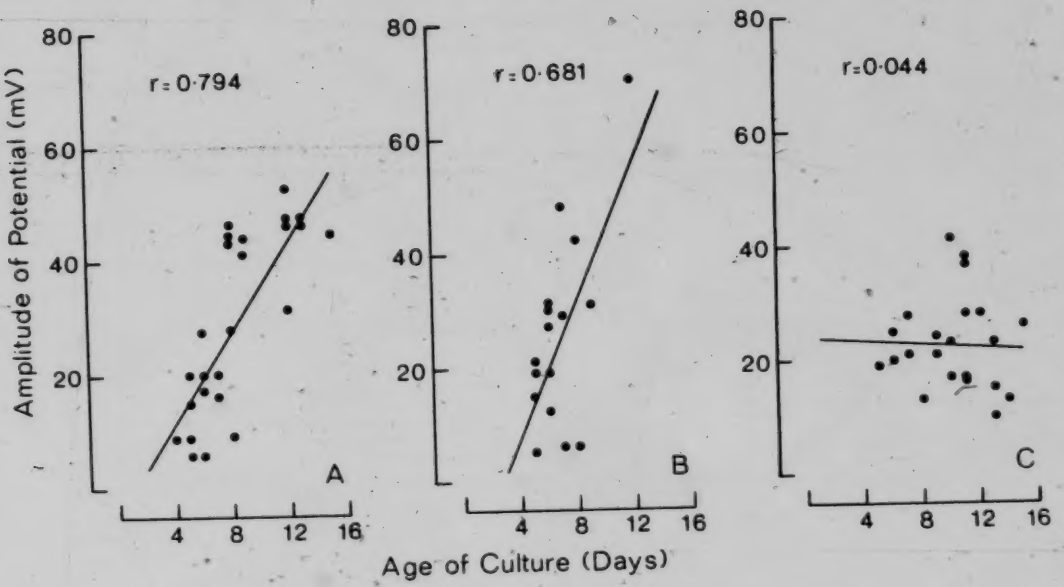


Fig. 7. Amplitude of regenerative potential correlated with age of control and 10^{-5}M ara-C treated cultures. a. control fibres n = 26. b. 36 hr ara-C treated fibres n = 16. c. 72 hr ara-C treated fibres n = 22. Each point was obtained from a single fibre. Data pooled from several cultures; lines calculaged by regression analysis with correlation coefficient (r) presented alongside.

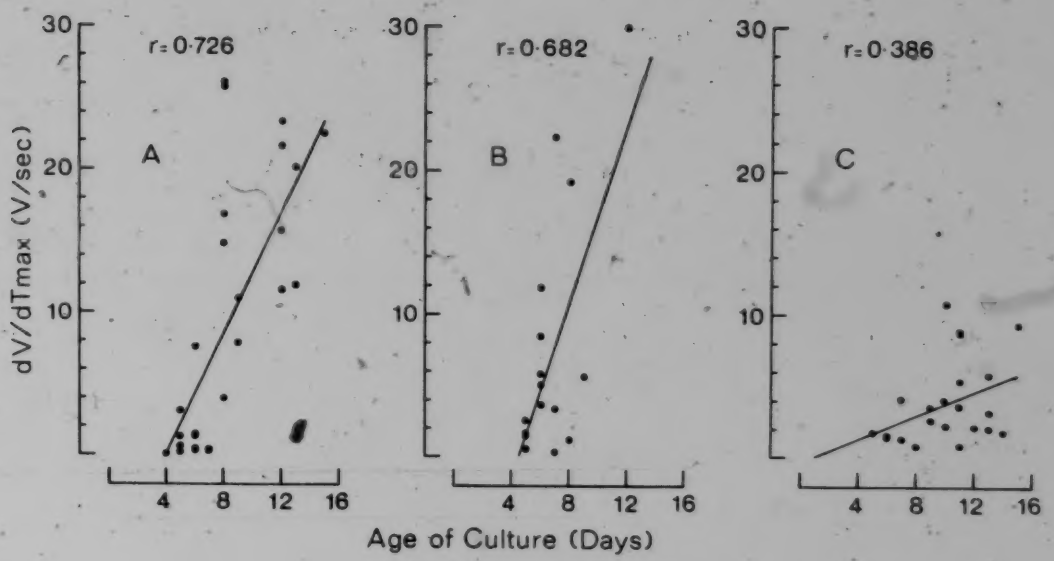


Fig. 8. Maximum rate of rise of regenerative potential correlated with age of control and 10^{-5}M ara-C treated cultures. a. control fibres n = 26. b. 36 hr ara-C treated fibres n = 16. c. 72 hr ara-C treated fibres n = 22. Each point was obtained from a single fibre. Data pooled from several cultures; lines calculated by regression analysis with correlation coefficient (r) presented alongside.

ara-C was 36 hours, both amplitude and dV/dt_{max} were well correlated with age of the myotubes (Figures 7B and 8B ; $0.001 < P < 0.01$ for both).

The mean E_m of the myotubes in both control and ara-C treated cultures which produced regenerative potentials in response to current injection was -70 mV. The mean threshold potential, the potential level to which the myotubes required to be depolarized before initiation of a regenerative potential, was -50 mV irrespective of treatment.

In older control and 36 hour treated cultures (8 days and over), spontaneous discharges were often observed on microelectrode penetration of the myotube membrane (Figure 9) presumably on account of damage sustained. The initial spike of such regenerative potentials was faster than regenerative potentials caused by electrical stimulation. The fate of the pacemaker depended on the apparent extent of damage inflicted on the cell membrane. In the examples illustrated both depolarization of the membrane to zero millivolts (9a) and stabilization of the membrane potential (9c) can be seen. These spontaneous potentials were never observed in the 72 hour treated cultures.

C. Discussion

The original use of ara-C was based on the premise that the effects of ara-C are restricted to those on the replication of DNA (Silagi, 1965; Cohen and Studzinski, 1967; Fischbach, 1972) and in myoblasts committed to differentiate and in myotubes no such replication of DNA occurs (Stockdale and Holtzer, 1961). Studies dealing with the effects of ara-C in muscle cultures have restricted themselves to the events at the time of myoblast fusion and have unquestioningly accepted the

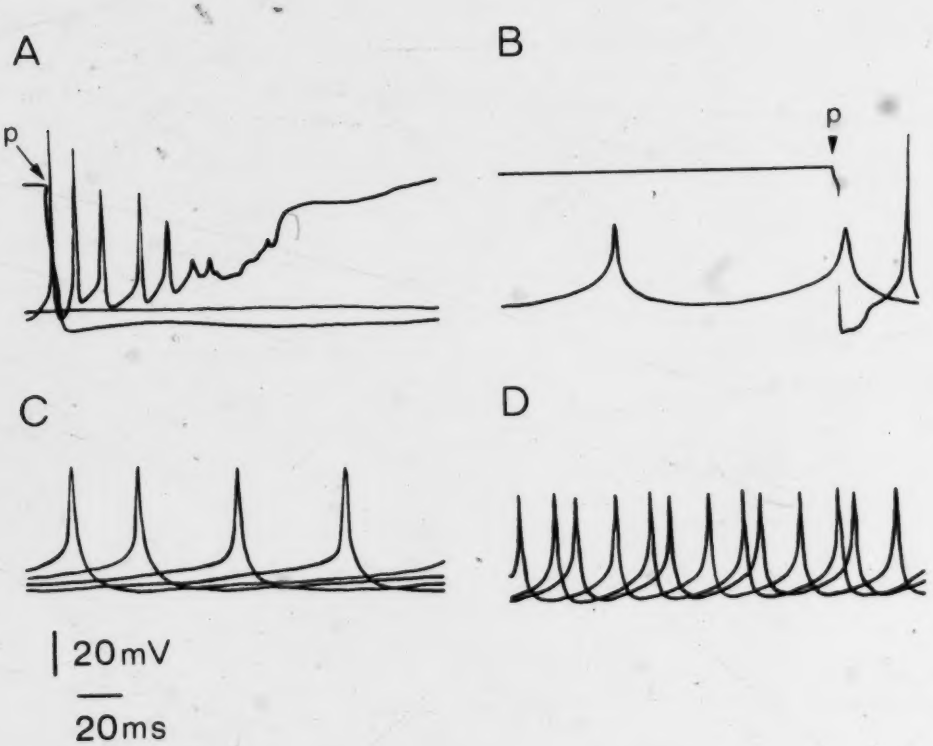
Fig. 9. Sample traces of spontaneous discharges from single fibres in control and 36 hr 10^{-5}M ara-C treated cultures.

(a) 12 day control fibre; E_m = 68 mV; dV/dT_{max} of initial spike = 90 V/sec; amplitude = 72 mV. Three consecutive oscilloscope sweeps depicting electrode penetration of membrane (p), followed by a falling membrane potential resulting in a burst of pacemaker activity terminated by the fall in membrane potential below 50 mV.

(b) 12 day 36 hr treated fibre; E_m = 60 mV; dV/dT_{max} of initial spike = 46 V/sec; amplitude = 75 mV. Two consecutive oscilloscope sweeps depicting electrode penetration of the membrane (p), followed by a depolarization and an initial rapid spike. This is succeeded by stable pacemaker activity comprising slower spikes of lower amplitude.

(c) 8 day control fibre; E_m = 76 mV; dV/dT_{max} = 24.5 V/sec; amplitude = 57 mV. Four consecutive oscilloscope sweeps depicting stable pacemaker activity resulting from a slow depolarization of the membrane.

(d) 12 day 36 hr treated fibre; E_m = 68 mV, dV/dT_{max} = 21.1 V/sec; amplitude = 48 mV. Three consecutive oscilloscope sweeps depicting stable pacemaker activity resulting from rapid depolarization of the membrane.


A
B
p
p
C
D
20mV
20ms

inhibition of DNA synthesis as the sole effect of ara-C (Doering and Fischman, 1974; Yeoh and Holtzer, 1977).

Other data would lead one to modify this view and the results presented indicate that differentiating myoblasts and myotubes are not completely unaffected by the drug despite the absence of active mitosis.

There is now general agreement that the action of ara-C is not restricted to an inhibition of thymidylate synthetase or indeed that this represents the principal cytotoxic effect (for review, see Creasy, 1975). Ara-C itself is undoubtedly incorporated into DNA for a time during *de novo* synthesis, and also into RNA. This latter pathway was not time dependent and continued throughout exposure to the drug (Chu, 1971). Incorporation initially occurred in lighter fractions of RNA, which coincidentally have a shorter half life, and spread to the heavier fractions during prolonged exposure. It is not unexpected, therefore, that selective inhibition of protein synthesis, particularly histones, has been reported in some cell lines (Borun, *et al.*, 1967; Schochetman and Perry, 1972).

Postfusion myotubes do not form a system in which active *de novo* synthesis of DNA occurs. Any DNA synthesis which occurs is presumably restricted to the repair of random breaks in the DNA chain (Stockdale and O'Neill, 1972). Ara-C in high doses (10^{-4}M) has been shown to antagonize DNA repair in replicating cells after ultraviolet induced damage (Hiss and Preston, 1977). The extent to which the inhibitor affects DNA repair in non-proliferating, non-traumatized cells such as muscle fibres is unknown, but it cannot be assumed that there is no adverse effect.

It is a dogma of genetics that the appearance of differentiated characteristics in cells and tissues is the manifestation of the activation of one or several genes, expressing themselves in the form of specific protein synthesis. The changes in resting membrane potential, time constant and regenerative potential which accompany maturation in muscle can be assumed to be the result of such genetic expression, and are effected by changes in the molecular architecture of the cell membrane as more proteins, many of them ionophores or ion channels, are assembled and incorporated in the phospholipid bilayer.

The differences which were noted were neither dramatic nor immediate. In general morphological development was unaffected and the development of electrophysiological parameters was, if anything, enhanced, judging by the more rapid increase in resting membrane potential over a four day period after treatment. This is in sharp contrast to the appearance of the replicating cells in the cultures at this time when cell rounding, detachment from the substratum and eventual cell death were clearly observable features and indicated that ara-C was exerting an undisputed cytotoxic effect on those cells engaged in the cell cycle. The retarded appearance of the regenerative potential and the lengthening of τ_m in treated cells together with the failure of fibres to maintain an elevated resting membrane potential indicated that ara-C does indeed exert an adverse effect and that the effect was not generally evident until after the removal of the drug. In addition, the severity of the effects was determined by the duration of exposure.

Since the nuclei in the myotubes had withdrawn from the cell cycle before exposure to ara-C, it is not likely that the compound was

incorporated in DNA in the process of normal replication. Furthermore, the delay in the manifestation of toxicity and the short half life of RNA argue against a significant role for incorporation in RNA as an explanation at least for the 36 hour exposed cultures. Similarly the known metabolites of ara-C would be rapidly leached from the myotubes after the removal of the drug from the medium and thus are unlikely to cause the effects noted (G.J. Lauzon and A.R.P. Paterson, Personal Communication). It must be admitted that in cultures exposed for 72 hours, the earlier appearance and greater extent of aberration from the control may indicate defective transcription during the period of exposure in addition to other effects. The role of incorporation of ara-C in DNA in the process of normal DNA repair has not been clearly defined in the available studies of the agent (see Creasy, 1975). However, it remains a reasonable possibility that some incorporation occurs by this means resulting in a proportion of defective genes in the total genetic pool of the multinucleate fibres. Thus the expression of a particular gene is unlikely to be completely inhibited, since all the nuclei in a fibre would not be similarly affected by the drug. Indeed the expression of a particular gene becomes reduced to a variable extent compatible with the observation of greater variance in input resistance.

An alternative explanation concerns the supporting role of fibroblasts in the overall maturation of muscle fibres. The use of ara-C inhibits or at least delays the formation by fibroblasts of a basement membrane. The relevance of this to electrophysiological development has not been established, but Lipton (1977) noted subsarcolemmal cytoplasmic densities associated with the extracellular deposition of

collagen fibrils in mixed myotube/fibroblast cultures. This morphological evidence can be adduced to support the view that myotube development is not wholly directed from within the myotube itself.

The use of ara-C was discontinued and breast muscle cultures were used for all further experiments, since fibroblasts overgrowth is much less and does not impede electrophysiological recording to the same extent as in leg muscle cultures.

IV. NORMAL DEVELOPMENT OF MEMBRANE PARAMETERS IN CHICK MUSCLE IN CULTURE

A. Introduction

In adult skeletal muscle resting membrane conductance (G_m) is principally determined by the contribution from both the potassium and the chloride ions, although resting membrane potential (E_m) is largely determined by potassium ion distribution and permeability. Since membrane potential is not constant during development, but rises throughout myogenesis from about -10 mV at myoblast fusion to attain mature levels over a period of several days, the ratio of ion conductances and their absolute values cannot be constant throughout this time. It was previously suggested that since the P_{Na}/P_K ratio was close to unity in myoblasts and declined to 0.43 several days after myoblast fusion, then membrane permeability to potassium rose during myogenesis and accounted for the rise in membrane potential (Dryden *et al.*, 1974). Before investigating potassium conductance, the development of E_m observed by previous workers (Dryden *et al.*, 1974; Spector and Prives, 1977) was confirmed, and the changes in G_m during myogenesis in culture were investigated.

B. Results

The control measurements from a number of cultures were combined to produce a pooled estimate of both E_m and G_m for pectoral muscle cultures. These are presented in Figures 10 and 11 respectively. The relatively large values for standard deviation reflect the variation between cultures when results are pooled.

Newly-formed breast myotubes had an initial mean resting membrane

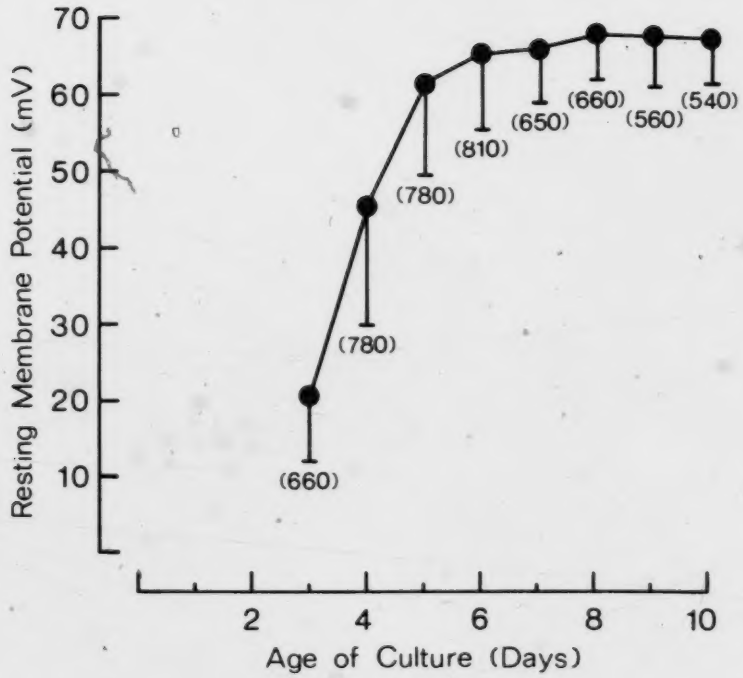


Fig. 10. Development of E_m in chick pectoral muscle in culture. Each point is the mean ± S.D. of the number of observations shown in parentheses. The results are pooled from between 18 and 27 separate cultures and this is reflected in the relatively large standard deviations.

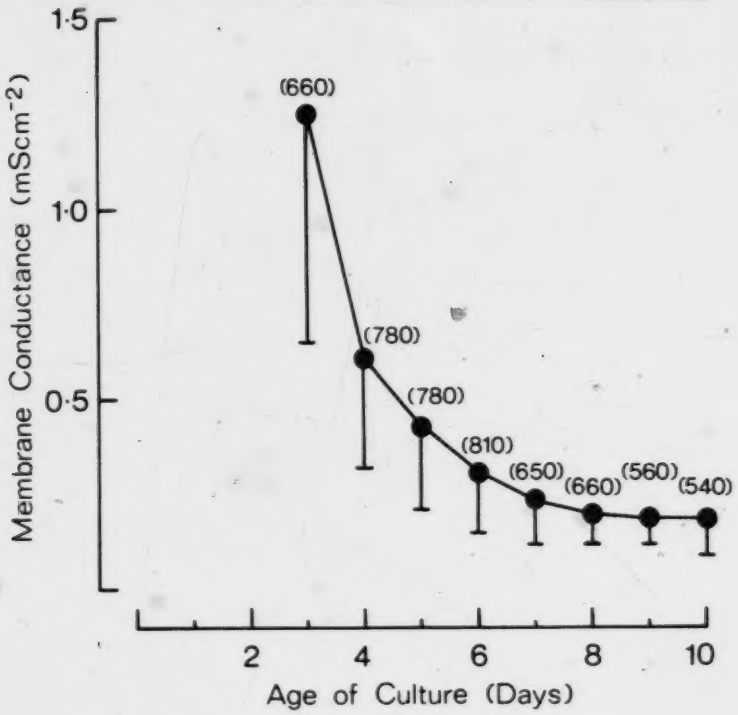


Fig. 11. The development of G_m in chick pectoral muscle in culture. Each point is the mean ± S.D. of the number of measurements shown in parentheses. The results are pooled from between 18 and 27 separate cultures, and this is reflected in the relatively large standard deviations. The fibres were the same as in Fig. 10.

potential of -21 mV. This is higher than that found by other workers in leg cultures (Dryden *et al.*, 1974; Fischbach *et al.*, 1971), however E_m was found to develop more rapidly in breast muscle by Spector and Prives, (1977). In this study, breast muscle E_m rose rapidly also over a 2-3 day period to reach -65 mV by day 6 in culture. Leg muscle cultures do not develop as rapidly as breast muscle and results from leg muscle experiments are included in table 6 as a comparison.

In the youngest myotubes, mean resting membrane conductance was found to be high initially (1.25 $mScm^{-2}$). Thereafter G_m fell rapidly to reach a level around 0.2 $mScm^{-2}$ (Figure 11). Both E_m and G_m were found to change most rapidly between days 3 and 5 in culture.

C. Discussion

The development of E_m observed by other workers was confirmed in this investigation and a more rapid development of pectoral muscle over thigh muscle was noted. This observation is consistent with published data (Dryden *et al.*, 1974; Fischbach, 1970; Spector and Prives, 1977) but does not seem to have been discussed. The difference in development of E_m between the two may be simply because the development of the pectoral muscle is more synchronous than that of the thigh. The lower mean E_m and larger S.D. values may simply reflect the fact that there is a greater variation in the rate of fusion and subsequent development of differentiated properties in the thigh muscle than in breast muscle, originating as it does from limb bud mesoderm rather than myotomal tissue.

No other similar developmental study in chick tissue has been pub-

TABLE 6

DEVELOPMENT OF E_m IN THIGH AND BREAST MUSCLE CULTURES OF THE CHICK

Age	Thigh E_m (mV) $\bar{x}$	S.D.	n	Breast E_m (mV) $\bar{x}$	S.D.	n
3	-	-	-	20.88	9.27	660 (22)*
4	32.95	15.95	110 (4)*	45.65	15.78	780 (26)
5	41.76	17.11	280 (11)	61.47	11.97	780 (26)
6	47.70	16.98	310 (12)	65.45	10.07	810 (27)
7	47.01	14.71	266 (9)	65.98	7.36	650 (22)
8	61.43	14.53	157 (7)	68.28	6.29	660 (22)
9	56.98	14.66	114 (5)	67.80	6.74	560 (19)
10	53.38	14.35	74 (3)	67.55	6.35	540 (19)
11	57.24	13.92	99 (4)	-	-	-

* Numbers in parentheses indicate number of cultures pooled.

lished but two studies have provided values from which G_m could be determined for comparison, and they reveal a considerable disparity. To take account of the elliptical nature of cultured myotubes, Harris *et al.* (1973) based their calculations on a notional "typical" myotube rather than deal with the dimensions of individual fibres and produced a specific membrane resistance which corresponded to a G_m of about 1.4 $mScm^{-2}$. Fischbach *et al.* (1971) used transient rather than steady state analysis, but their measurements are similar to those found in five day fibres in the present study. Ritchie and Fambrough (1975) performed a developmental study of the passive electrical properties of rat muscle in culture from which values for G_m ranging from 1.04 $mScm^{-2}$ at day 3 to 0.638 $mScm^{-2}$ at day 9 can be derived. Taking into account the relatively slower rates of growth of mammalian muscle in culture, these values are in broad agreement with the present data, and certainly confirm the trend to lower membrane conductance with myotube maturation.

A comparison with values for G_m in intact muscle (see Table 7) reveals that the values for mature myotubes are similar to those published for frog muscle and for chicken ALD but not chicken PLD or rat or goat. This is of some interest since the ALD is a multiply innervated muscle while the PLD, and mammalian muscles are focally innervated. Membrane conductance falls after denervation (Albuquerque and Thesleff, 1968; Albuquerque and McIsaac, 1970) and the low G_m in the cultured myotubes and in chicken ALD may represent lack of or different neurotrophic control.

The observation that young chick myotubes have a high membrane conductance which declines during the maturation process is in contrast to

TABLE 7

ESTIMATES OF SKELETAL MUSCLE MEMBRANE CONDUCTANCE IN VARIOUS SPECIES

Reference	Species	G_m ($mScm^{-2}$)
Fink & Lüttgau (1976)	Frog	0.320
Hodgkin & Horowicz (1959b)	Frog	0.255
Mashima & Washio (1964)	Frog	0.267
Stanfield (1970)	Frog	0.283
Stanfield (1970)	Frog	0.298
Fedde (1969)	Chick (ALD)	0.228
Fedde (1969)	Chick (PLD)	1.782
Warnick & Albuquerque (1979)	Chick (PLD)	1.428
Harris *et al.* (1973)	Chick (explant culture)	1.388
Fischbach *et al.* (1971)	Chick (primary culture)	0.379*
Morgan *et al.* (1975)	Pigeon	3.142
Lorković & Tomanek (1977)	Rat (gastrocnemius)	0.944
Lorković & Tomanek (1977)	Rat (soleus)	1.186
Camerino & Bryant (1976)	Rat	3.456
Palade & Barchi (1977a)	Rat	2.450
Ritchie & Fambrough (1975)	Rat (3 day primary culture)	1.040
Ritchie & Fambrough (1975)	Rat (9 day primary culture)	0.638
Powell & Fambrough (1973)	Mouse (primary culture)	1.441
Powell & Fambrough (1973)	Mouse (primary culture)	0.478*
Bryant & Morales-Aguilera (1971)	Goat	0.702
Bryant & Morales-Aguilera (1971)	Goat	0.657
Bryant & Camerino (1976)	Goat	0.951

* Transient analysis

earlier predictions (Dryden *et al.*, 1974). These predictions were based on the calculation that both sodium and potassium had equal permeabilities in myoblasts, and that the permeability ratio changed in favour of potassium with increasing membrane potential. The relative insensitivity of young myotubes to varying external concentrations of potassium suggested that potassium might be relatively impermeant at that stage, and that P_K increased with development causing the membrane potential to rise towards the potassium equilibrium potential. It is clear that this view is no longer tenable and that an alternative explanation for rising E_m must be sought.

Changes in membrane conductance as a function of differentiation are not unique to skeletal muscle fibres. Pappano and Sperelakis (1969) reported that the input resistance of isolated single heart cells in culture was three times that measured in clusters or monolayers where the cells had formed contacts and were capable of rhythmic beating. Here, of course, changes in surface area of the functional syncytium may in part provide the explanation for the observed change in R_{in}, but no data to support this was presented. In the present work measurements were restricted to myotubes due to the difficulty in obtaining successful multiple penetrations of myoblasts. The situation prior to fusion is therefore unknown but after fusion, presumably the analogue of cell/cell contact in heart muscle culture, the sequence of development seems opposite to that noted by Pappano and Sperelakis (1969) in that specific membrane resistance increases with development of skeletal muscle.

V. EFFECTS OF AGENTS REPUTED TO AFFECT POTASSIUM CONDUCTANCE

A. Introduction

Potassium and chloride are the two ions which principally determine the resting membrane conductance of adult skeletal muscle. When chloride in the bathing solution is replaced by impermeant anions, the contribution from sodium is usually small enough to be neglected. Values for $\bar{g}K$ obtained in this way range from 0.1 $mScm^{-2}$ in frog, where it accounts for 33% of the total membrane conductance (Hodgkin and Horowicz, 1959b; Mashima and Washio, 1964) to 0.1 - 0.3 $mScm^{-2}$ in mammals where $\bar{g}K$ apparently contributes only 11 - 18% of total G_m (Bryant and Morales-Aguilera, 1971; Bryant and Camerino, 1976; Lorković and Tomanek, 1977; Palade and Barchi, 1977a). The suggestion that membrane permeability to potassium rises during myogenesis (Dryden *et al.*, 1974) was investigated by measuring the depression in membrane conductance caused by the exposure of cultures to tetraethylammonium (TEA) and caesium, two agents reported to block selectively $\bar{g}K$ (Narahashi, 1974; Adelman and French, 1978). TEA suffers from the drawback that it is not very potent and relatively high concentrations are required (10-100 mM) (Narahashi, 1974). However, various workers have reported that certain aminopyridines are also selective blockers of potassium channels at lower concentrations than TEA (Pelhate and Pichon, 1974; Gillespie and Hutter, 1975; Bowman *et al.*, 1976; Schauff *et al.*, 1976; Yeh *et al.*, 1976a; Ulbricht and Wagner, 1976; Meves and Pichon, 1977; Kirsch and Narahashi, 1978). The two most potent analogues are 3,4-diaminopyridine (3,4-DAP) and 4-aminopyridine (4-AP). The effects of these two drugs on the development of G_m were also investigated in an attempt to confirm the findings with TEA and caesium.

B. Results

1. TEA and Caesium

Figures (12 and 13) illustrate the effect of addition of 10 mM TEA to BSS. At all stages of development depolarization occurred and G_m was reduced. This reduction was 0.2 $mScm^{-2}$ on day 3 but on day 4 and at all subsequent stages of development G_m was reduced by about 0.1 $mScm^{-2}$ in the presence of TEA. When 100 mM sodium chloride in the BSS was replaced by 100 mM TEA chloride the reduction in G_m was identical to that observed with 10 mM TEA, indicating that 10 mM TEA produced the maximum change in G_m.

Figures 14 and 15 show the effect on E_m and G_m of replacement of potassium in BSS by caesium (5.369 mM). Again depolarization occurred at all stages of development. Caesium produced a similar depression of G_m to that observed with TEA, i.e. on day 3 G_m was reduced by 0.2 $mScm^{-2}$ but on day 4 and subsequently G_m was reduced by about 0.1 $mScm^{-2}$.

2. 4-AP and 3,4-DAP

The effects of 10^{-3}M 4-AP on the development of E_m and changes in membrane conductance (G_m) are shown in Figures 16 and 17. 4-AP depressed E_m at all stages of development in a similar manner to that found previously with TEA and caesium. However, in contrast to the other two agents, 4-AP caused an increase in G_m of 0.15 $mScm^{-2}$ in young fibres from days 3 to 5 of culture. On day 6 and each subsequent day the addition of 4-AP caused a reduction in G_m of about 0.1 $mScm^{-2}$. The experiment was repeated with the more potent analogue, 3,4-DAP and the results, which are similar to those of 4-AP, are illustrated in Figures 18 and 19.

Membrane potential was also depressed by 10^{-4}M 3,4-DAP at all stages

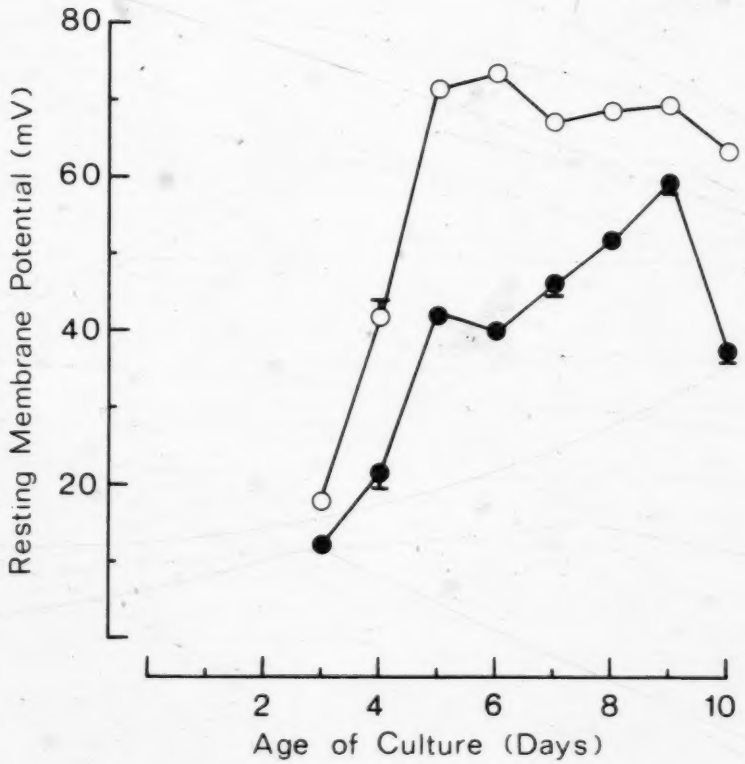


Fig. 12. Effect of TEA on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same 30 cells were then measured 15 minutes after changeover to 10 mM TEA medium (●). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control at $P < 0.001$ using Student's paired *t*-test.

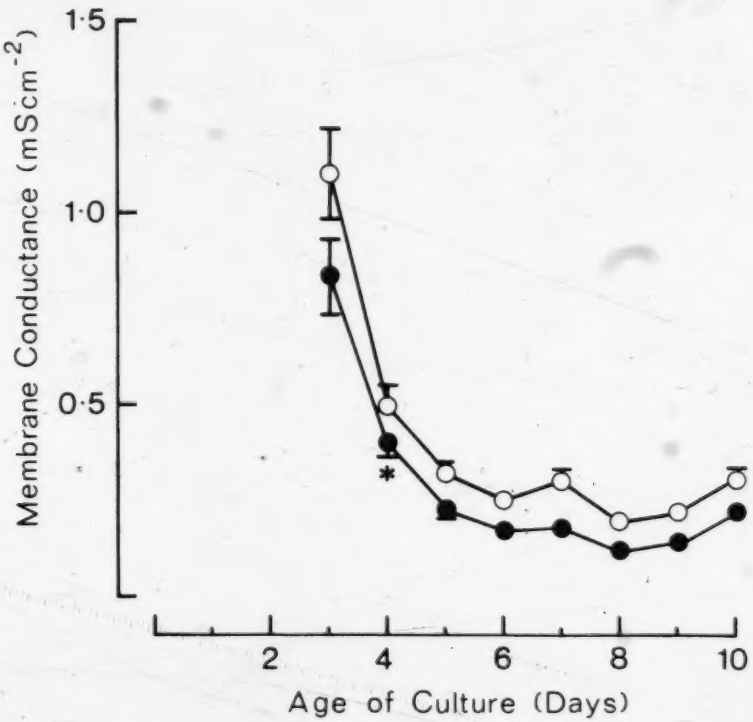


Fig. 13. Effect of TEA on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to 10 mM TEA medium. Each point is the mean ± SEM of 30 measurements. Each test point is significantly different from the control at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. The fibres were the same as in Fig. 12. * $P < 0.005$.

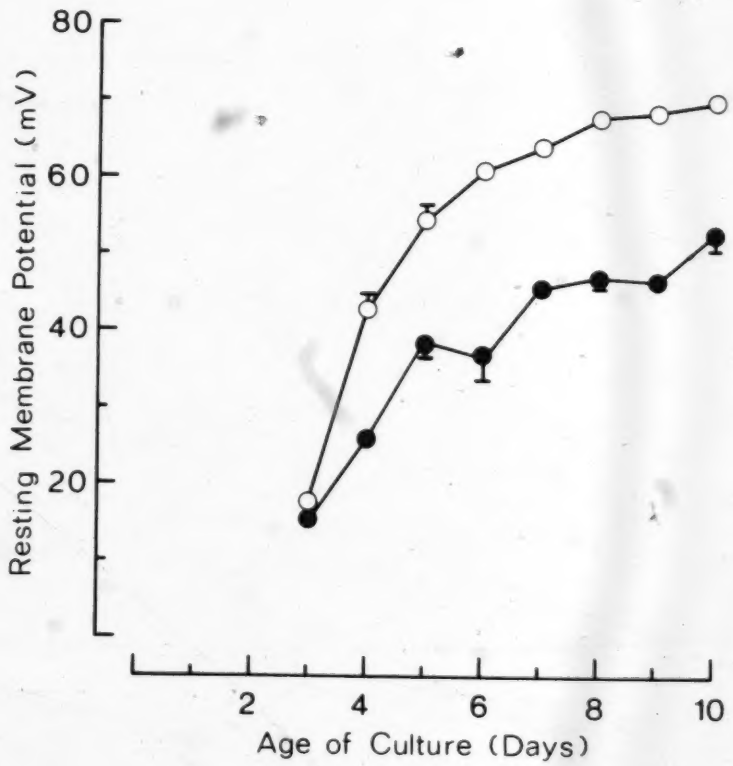


Fig. 14. Effect of replacement of external potassium with caesium on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were measured 15 minutes after changeover to potassium free medium containing 5.369 mM caesium (●). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from control at $P < 0.001$ using Student's paired *t*-test.

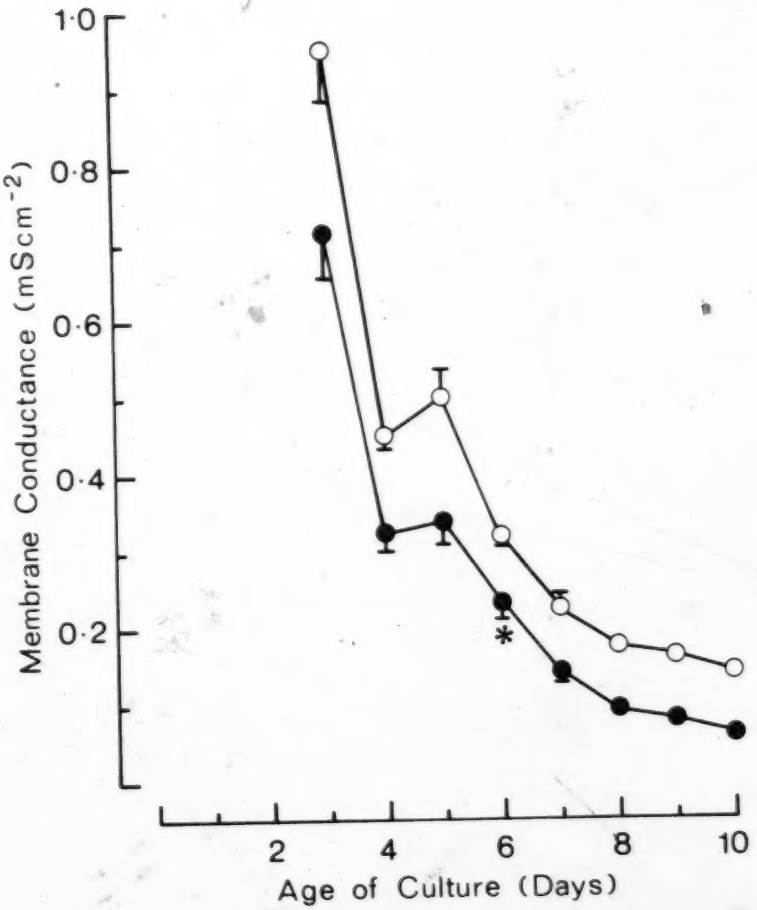


Fig. 15. Effect of replacement of potassium with caesium on G_m during development of chick skeletal muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to potassium free medium containing 5.369 mM caesium. Each point is the mean ± SEM of 30 measurements. Each test point is significantly different from control at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. The fibres were the same as in Fig. 14. * $P < 0.005$.

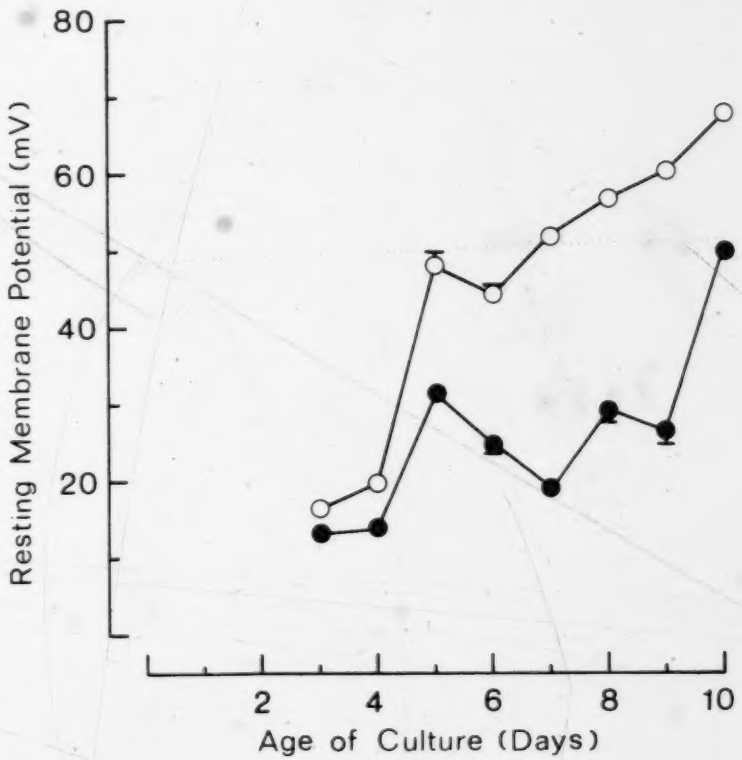


Fig. 16. Effect of 4-AP on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same 30 cells were then measured 15 minutes after changeover to 10^{-3}M 4-AP medium (●). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control at $P < 0.001$ using Student's paired *t*-test.

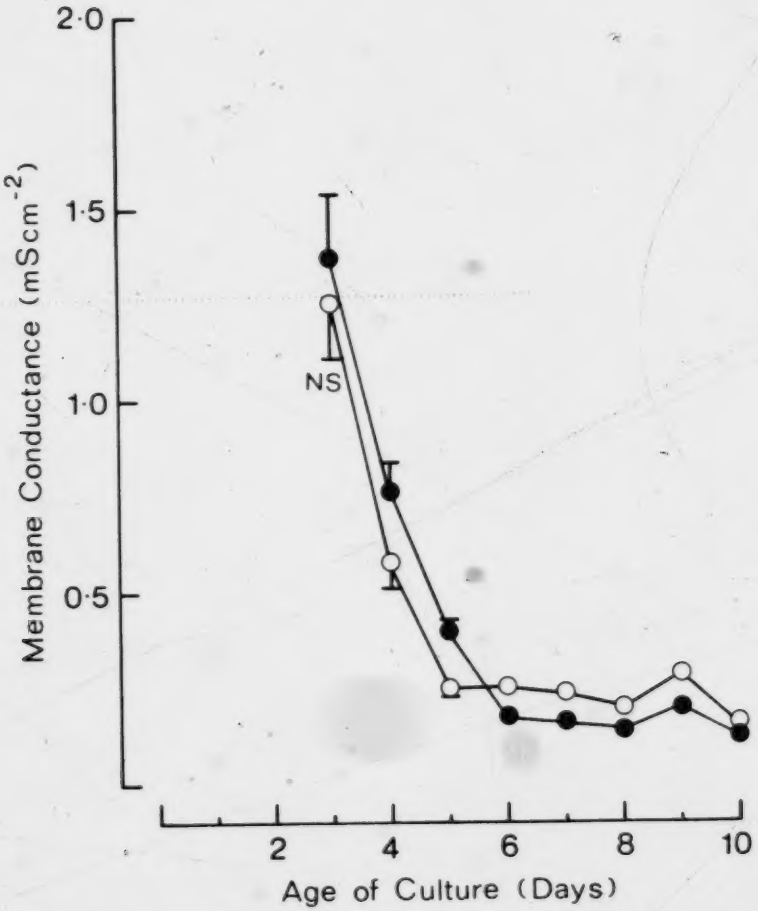


Fig. 17. Effect of 4-AP on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to 10^{-3}M 4-AP medium. Each point is the mean ± SEM of 30 measurements. Each test point is significantly different from the control at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. The fibres were the same as in Fig. 16.

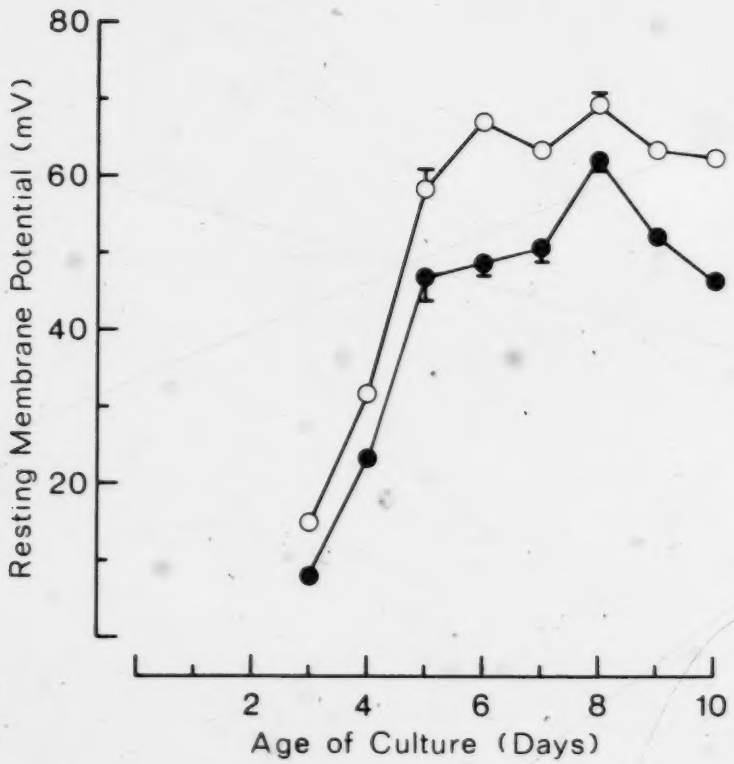


Fig. 18. Effect of 3,4-DAP on E_m during development of chick skeletal muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were measured 15 minutes after changeover to 10^{-4}M 3,4-DAP medium (●). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control at $P < 0.001$ using Student's paired *t*-test.

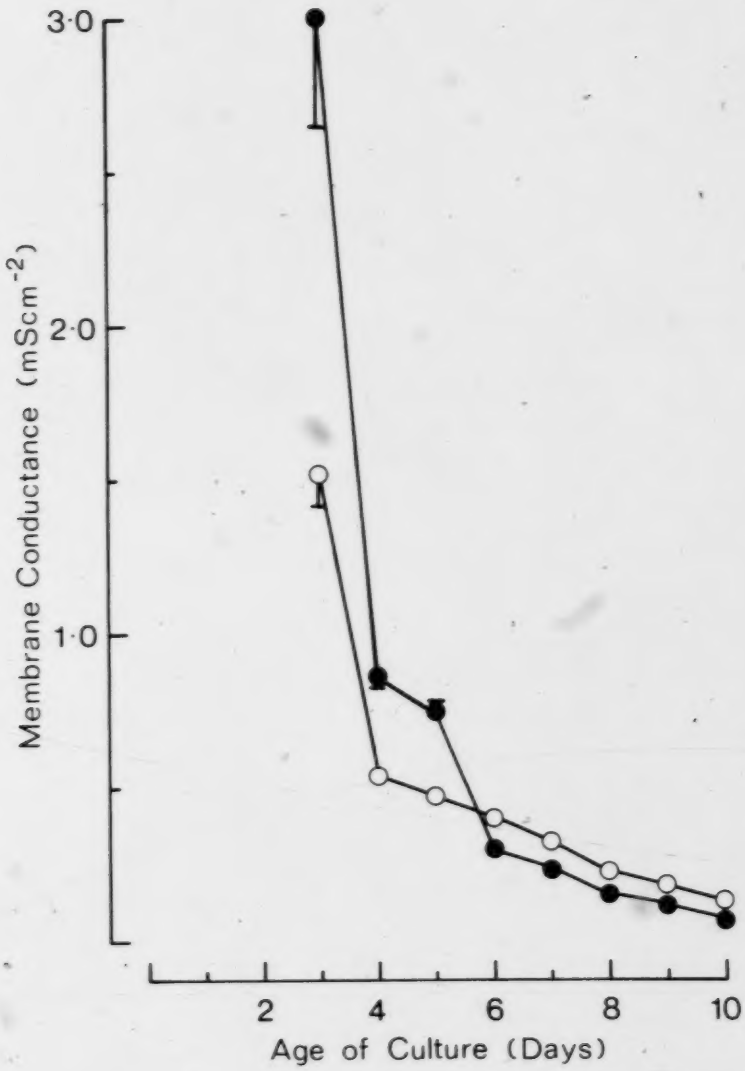


Fig. 19. Effect of 3,4-DAP on G_m during development of chick skeletal muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to 10^{-4}M 3,4-DAP medium. Each point is the mean ± SEM of 30 measurements. Each point is significantly different from the control at $P < 0.001$ using Student's paired *t*-test. The fibres were the same as in Fig. 18.

of development but the amount of depolarization was less than observed with 4-AP (Figure 16). The effects of 3,4-DAP on membrane conductance were qualitatively similar to that observed with 4-AP. However, the initial increase in G_m (1.5 $mScm^{-2}$) on day 3 was ten times that found with 4-AP and twice that found earlier on days 4 and 5 (0.3 $mScm^{-2}$). On day 6, 3,4-DAP reduced G_m by 0.1 $mScm^{-2}$ from control levels. This reduction was maintained throughout the remainder of the experiment although by day 10 the depression of G_m had fallen to 0.05 $mScm^{-2}$. The depression of G_m observed in the older fibres with both aminopyridines is of a similar magnitude to that found with maximally effective concentrations of either TEA or caesium.

Combination experiments with 4-AP and TEA were performed to investigate whether the two agents were additive or not. The same results were observed irrespective of the addition sequence and the results obtained with 4-AP pretreatment prior to 4-AP in combination with TEA are illustrated in Figures 20 and 21. Both 4-AP and TEA alone cause depolarization and when used in combination a further fall in E_m was observed.

In this particular experiment 4-AP increased G_m by 1 $mScm^{-2}$ on day 3, 0.3 $mScm^{-2}$ on day 4 and 0.15 $mScm^{-2}$ on day 5 which is similar to the results observed with 3,4-DAP. The addition of 10 mM TEA to the 4-AP treated fibres caused a reduction in G_m of about 0.5 $mScm^{-2}$ on day 3 and 0.2 $mScm^{-2}$ on days 4 and 5. The fall in G_m on day 3 caused by the combination was not significantly different from the level observed in 4-AP alone due to the large variation in measurements which was seen in young fibres. On days 4 and 5, the membrane conductance in the presence of the combination was significantly less than that observed with 4-AP

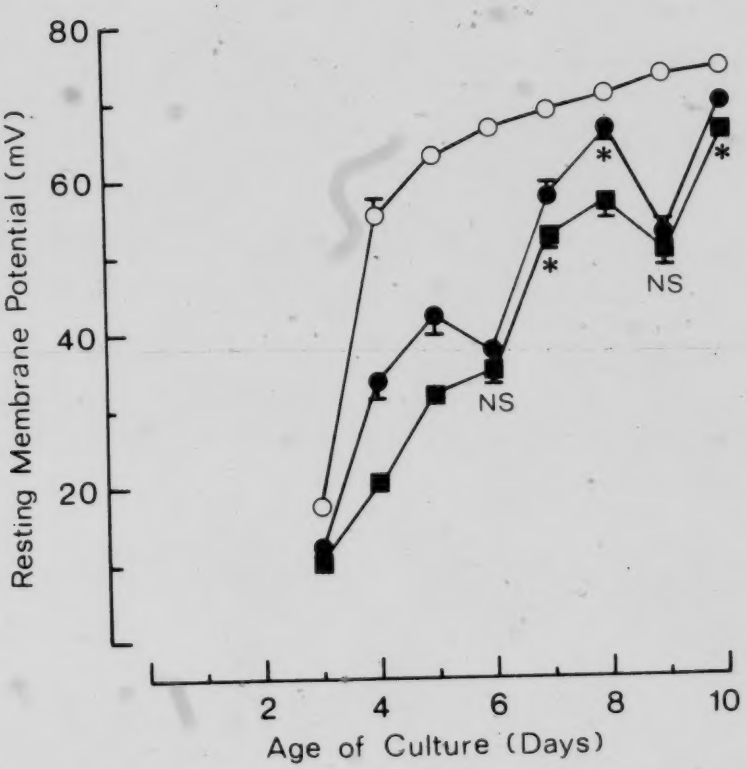
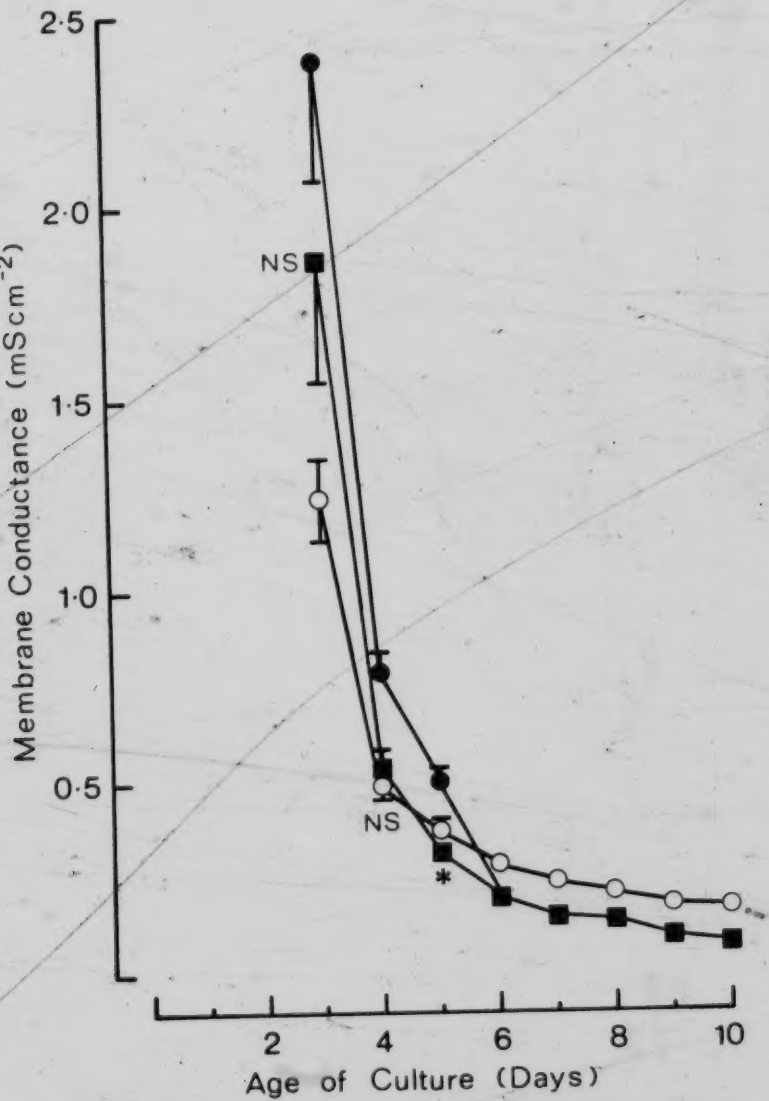


Fig. 20. Effect of 4-AP and then 4-AP plus TEA on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to 10^{-3}M 4-AP medium (●). The same fibres were then tested again 15 minutes after changeover to 10^{-3}M 4-AP plus 10 mM TEA medium (■). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. * $P < 0.02$.

Fig. 21. Effect of 4-AP and then 4-AP plus TEA on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values were measured in the same cells 15 minutes after changeover to 10^{-3}M 4-AP medium (●). The same fibres were then tested again 15 minutes after changeover to 10^{-3}M 4-AP plus 10 mM TEA medium (■). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. * $P < 0.01$. The fibres were the same as in Fig. 20.


2·5
2·0
1·5
1·0
0·5
Membrane Conductance (mScm^{-2})
NS
NS
*
2
4
6
8
10
Age of Culture (Days)

alone ($P < 0.001$) and on day 5 G_m fell significantly below the value of control measurements ($P < 0.01$). On day 6 and subsequently, membrane conductance was reduced in the presence of 4-AP alone by 0.1 $mScm^{-2}$ but the addition of 10 mM TEA caused no further alterations in G_m and the values measured in 4-AP plus TEA are superimposed on those measured in 4-AP alone.

C. Discussion

Since the methods of measurement required repeated penetration of the same muscle fibre with the microelectrode, it was important to ensure that membrane damage was not extensive and was rapidly repaired. In preliminary experiments control fibres were penetrated several times without alteration in measured R_{in} or loss of membrane potential. Where damage did occur, it was immediately evident under the microscope since blebs of phase-dense cytoplasm could be seen to leak from the cell into the surrounding medium while both E_m and R_m declined to zero. Fibres which exhibited this phenomenon were rejected. The changes in either E_m or R_m which were observed were therefore attributable to the agents which had been used, and were not due to artifacts arising from the techniques.

All the agents used to estimate resting potassium conductance in the experiments produced a significant depolarization of the fibres at all stages of development. Since the depolarization caused by TEA and caesium is accompanied by a fall in G_m it is reasonable to associate the two phenomena as effect and cause. As the potassium conductance is reduced, the P_{Na}/P_K ratio reverts towards that found in myoblasts and

immature myotubes, producing the observed reduction in E_m as predicted by the Goldman-Hodgkin-Katz equation. However, in the case of the aminopyridines, the membrane depolarization is accompanied by an increase in G_m in young fibres. Thus, the mechanism of myotube depolarization brought about by the aminopyridines in the first five days of culture must differ from that caused by either TEA or caesium. Although several groups of workers have reported no depolarization with the aminopyridine analogues (Schauff *et al.*, 1976; Pelhate and Pichon, 1974; Molgó *et al.*, 1977, 1979; Shapovalov and Shiriaev, 1978), in these reports lower concentrations of aminopyridines were used (10^{-6} - 10^{-4}M) and it is evident that maximum blockade is not attained until 10^{-3}M (4-AP) or 10^{-4}M (3,4-DAP) (Yeh *et al.*, 1976b; Kirsch and Narahashi, 1978). Also, the facilitatory action of the aminopyridines on neuromuscular transmission is seen only at close to millimolar concentrations.

Depolarization of excitable cells seems to be generally observed in a number of species and tissues. Yeh *et al.* (1976a) observed depolarization and repetitive firing in squid axons exposed to 10^{-3}M 4-AP as did Llinás *et al.* (1976) using millimolar concentrations of 3-AP. Similar results were obtained in this tissue with 10^{-4}M 3,4-DAP (Kirsch and Narahashi, 1978). Depolarization of the sixth ganglion of the cockroach occurred with concentrations of 4-AP higher than 5×10^{-5}M (Hue *et al.*, 1975) and depolarization with increased reflex activity has also been reported in the frog and cat spinal cord preparations in response to 4-AP (Galindo and Rudomín, 1978; Jankowska *et al.*, 1977). Repetitive firing was also observed at the frog neuromuscular junction in the presence of 10^{-5}M 4-AP (Horn *et al.*, 1979).

In contrast to the above reports, and the present observations, Bowman *et al.* (1977) observed no depolarization in cultures of chick leg muscle exposed to 10^{-3}M 4-AP. The reasons for this discrepancy are not obvious since it seems improbable that breast muscle in culture is fundamentally different from leg muscle. However, the authors made random measurements and compared population means before and 10 minutes after addition of 4-AP in contrast to the methods used in the present work where the same muscle fibres were examined before and 15 minutes after addition of the drug.

TEA and caesium both reduce resting membrane conductance to similar extents during myogenesis in culture. Since caesium is used to replace external potassium in the BSS then the reduction in G_m in the presence of caesium is due to the loss of steady-state potassium conductance. Because 100 mM TEA caused no greater effect on G_m than 10 mM TEA and this concentration of TEA produces similar changes in G_m to caesium, this suggests that 10 mM TEA is maximally blocking only potassium channels, and that it has no effect on other ion channels present. TEA is required in relatively high concentrations to block potassium currents in other excitable tissues (Narahashi, 1974). This is probably because both TEA and caesium are competing with the potassium ion at the entrance to the channel, rather than by binding to specific receptors at or near to the channel.

The potassium conductance of cultured chick skeletal muscle is relatively constant at 0.1 $mScm^{-2}$ during myogenesis and does not rise with the developing membrane potential. The relative contribution from potassium to total membrane conductance is initially 25% and tends to

increase with maturation to 30-35%. This more closely approximates results in amphibia rather than mammals. No data from cultured muscle or adult chick muscle is available although Morgan *et al.* (1975) found that in adult pigeon $\bar{g}K$ was 18% of G_m.

Since $\bar{g}K$ remains unchanged during myotube development at about 30% of G_m but membrane conductance falls, then the conductance to other ions must diminish. While calcium is possibly one such ion, large inflows of calcium have been considered to be toxic to these cells (Dryden and Thomson, 1979). Principal candidates are therefore the chloride and sodium ions. Work presented subsequently in this thesis (Chapter VI) indicates that chloride conductance does indeed decrease with maturation of muscle fibres in culture, but since chloride appears to be passively distributed across the cell membrane in response to the prevailing E_m at all times, this change in conductance cannot explain the increase in E_m with fibre development. One is therefore left to conclude that there is a significant resting sodium conductance in young myotubes, at least equal to that of potassium to provide a P_{Na}/P_K ratio of 1.0, and that this resting sodium conductance declines with the acquisition by the myotube of more differentiated characteristics. Further data to support this view are presented in Chapter VIII.

In older cultures, both 3,4-DAP and 4-AP reduced the membrane conductance by an extent similar to that seen when external potassium was replaced with caesium, or when a maximally blocking concentration of TEA was used. Furthermore, since the addition of a maximally blocking concentration of TEA to 4-AP treated fibres did not alter G_m further, it would appear that in these fibres the aminopyridines block potassium

channels selectively.

However, in immature muscle fibres the aminopyridines increased G_m and the action of the drugs in these fibres cannot solely be blockade of potassium conductance. It is possible that any blockade of $\bar{g}K$ is masked by an increased conductance elsewhere, but since TEA causes G_m to fall after 4-AP treatment, it is more likely that the potassium channel remains unblocked in the presence of the aminopyridines before the sixth day of development.

This illustrates the difference between a drug which is active in binding to a receptor, and an agent which acts more as an impermeant ion, attracted to binding sites within the channel, but incapable of completing the passage through the channel by virtue of its excessive diameter. Both TEA and caesium are thought to occlude the potassium channel physically in this latter fashion (Armstrong, 1975; Adelman and French, 1978) whereas the aminopyridines are believed to produce blockade after interacting with a receptor site at or near the potassium channel. The nature of this receptor and the mode of blockade is subject to controversy (Gillespie and Hutter, 1975; Ulbricht and Wagner, 1976; Yeh *et al.*, 1976b; Meves and Pichon, 1977; Gillespie, 1977). From these reports, it is not entirely clear whether the inactivation gate of the potassium channel is the site of action of the aminopyridines, but it is certain that the effect of the drugs is lost when the membrane is first depolarized, and potassium channels are opened according to Hodgkin-Huxley kinetics (Hodgkin and Huxley, 1952d). Since the membrane potential of immature muscle fibres is depressed, it is entirely possible that the failure of the aminopyridines to block potassium channels in these cells

is related to the "activated" state of the channels, although no clear evidence has been presented that activation or deactivation can be induced in the potassium channels of immature muscle.

The failure of the aminopyridines to bind to their receptor at the potassium channel of immature myotubes cannot explain, however, the observed increase in G_m accompanied by a depolarization. In this case an additional mechanism must be adduced implicating either sodium or calcium ions, or both. This mechanism is apparently lost during development and is not longer detectable after five days of culture. Further evidence (Chapter IX) suggests that sodium conductance is increased by the application of these drugs to immature muscle fibres. It is entirely possible that the membranes of such cells may not be unique in possessing a sodium mediated response. While the mechanisms of $\bar{g}K$ blockade produced by TEA and caesium on the one hand and the aminopyridines on the other may well differ (Yeh *et al.*, 1976b; Harvey and Marshall, 1977; Fink and Wettwer, 1978; Kenyon and Gibbons, 1979) the differences in action noted in *Aplysia* neurones (Klee, 1978) and in the facilitation of neuromuscular transmission (Lundh *et al.*, 1977) may be attributable to increased sodium conductance as well as depressed $\bar{g}K$ at locations such as the nerve terminal. In the case of immature muscle fibres, such an increase in $\bar{g}Na$ would adequately explain the observed depolarization of the membrane.

VI. EFFECTS OF AGENTS REPUTED TO AFFECT CHLORIDE CONDUCTANCE

A. Introduction

In adult frog muscle chloride contributes two thirds of the total resting membrane conductance (Hodgkin and Horowicz, 1959b; Mashima and Washio, 1964; Stanfield, 1970; Fink and Lüttgau, 1976). In mammalian and avian muscle, the contribution by chloride to G_m is even greater - between 82 and 90% (Morgan *et al.*, 1975; Bryant and Camerino, 1976; Camerino and Bryant, 1976; Lorković and Tomanek, 1977; Palade and Barchi, 1977a). In contrast, chloride appeared to be relatively impermeable in primary cultures and in the clonal line (L6) of rat muscle (Ritchie and Fambrough, 1975; Kidokoro, 1975a). Chloride conductance was not measured in either case but membrane potential and input resistance were little affected by a sudden reduction in external chloride concentration.

The portion of the total membrane conductance in chick muscle cultures due to chloride was estimated in the experiments reported in this chapter by several methods. Primarily chloride was substituted by sodium isethionate and potassium methylsulphate and the difference between the two estimates of G_m was taken to be due to the chloride ion. Several agents have been reported to block specifically chloride conductance in adult skeletal muscle and two of these were tested in this system: *viz.* anthracene-9-carboxylic acid and zinc chloride (Bryant and Morales-Aguilera, 1971; Palade and Barchi, 1977b; Mashima and Washio, 1964; Hutter and Warner, 1967c).

B. Results

Zinc chloride was used at a concentration of 0.5 mM (Mashima and Washio, 1964) and buffered with 5 mM sodium PIPES (Woodbury and Miles, 1973) (Solution F). A 10^{-3}M solution of the sodium salt of anthracene-9-carboxylic acid was prepared in 1% sodium bicarbonate and this was added to BSS to yield a final concentration of 5×10^{-5}M (Bryant and Morales-Aguilera, 1971).

When external chloride was replaced by impermeant anions (Solution E), membrane potential was significantly depressed at all stages of development (Figure 22). A similar depolarization was seen with 0.5 mM $ZnCl_2$, but in 5×10^{-5}M 9-AC only young fibres were depolarized to the same extent. The effect of 9-AC on membrane potential progressively diminished as the cultures matured until by day 10 in culture there was no significant difference in E_m between control and 9-AC treated fibres.

Both zinc and 9-AC impaired membrane integrity in young cultures (days 3 and 4 and to a lesser extent, day 5). After 30 minutes exposure to 0.5 mM $ZnCl_2$ cells were seen to round up and detach from the bottom of the culture dish. Blebs of phase dense cytoplasm formed above the fibres which had not yet detached and no membrane potential could be recorded from such fibres (Figure 23). Similar but less pronounced effects occurred on exposure to 9-AC (after 60 mins) but there was no evidence of membrane damage in fibres exposed to zero chloride solution (Figure 23). The inclusion of 10^{-6}M verapamil hydrochloride in the medium prevented membrane rupture and leakage of cytoplasm for at least 90 minutes in the presence of either zinc or 9-AC.

When the effects of zinc on membrane conductance were examined,

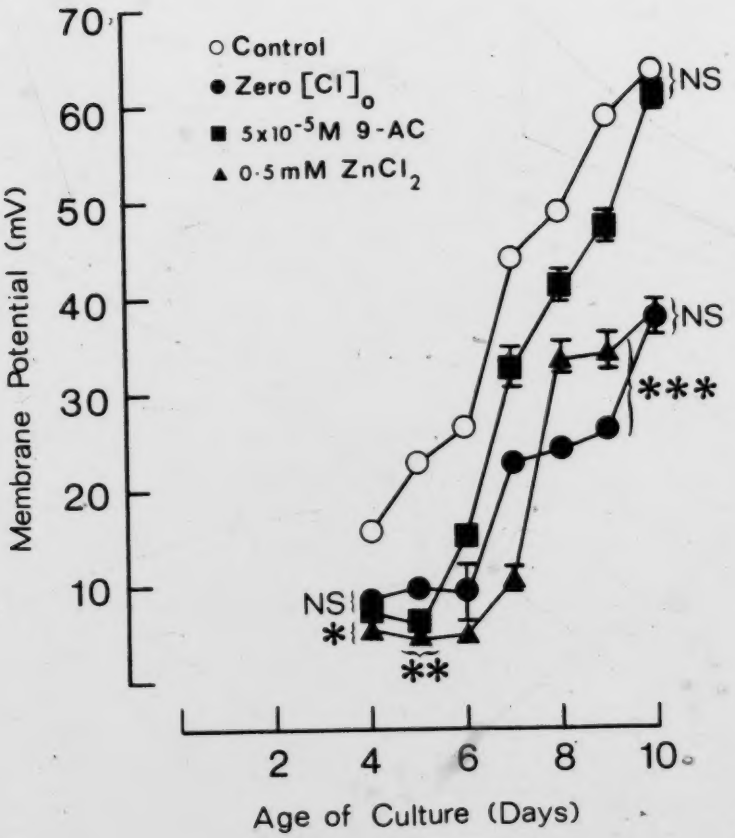


Fig. 22. Effect of removal of external chloride or the application of 9-AC or $ZnCl_2$ on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to zero $[Cl]_o$ (●), 5 x 10^{-5}M 9-AC (■) or 0.5 mM $ZnCl_2$ (▲) medium. Each point is the mean ± SEM of at least 20 observations. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired or unpaired *t*-test) unless otherwise indicated. * $P < 0.05$, ** $P < 0.025$, *** $P < 0.005$.

Fig. 23. Effect of removal of external chloride, 9-AC or $ZnCl_2$ on membrane integrity of developing myotubes in chick muscle cultures.

A. 3 day old fibres incubated in BSS for 60 minutes.

B. 3 day old fibres incubated in zero $[Cl]_o$ (Solution E) for 60 minutes.

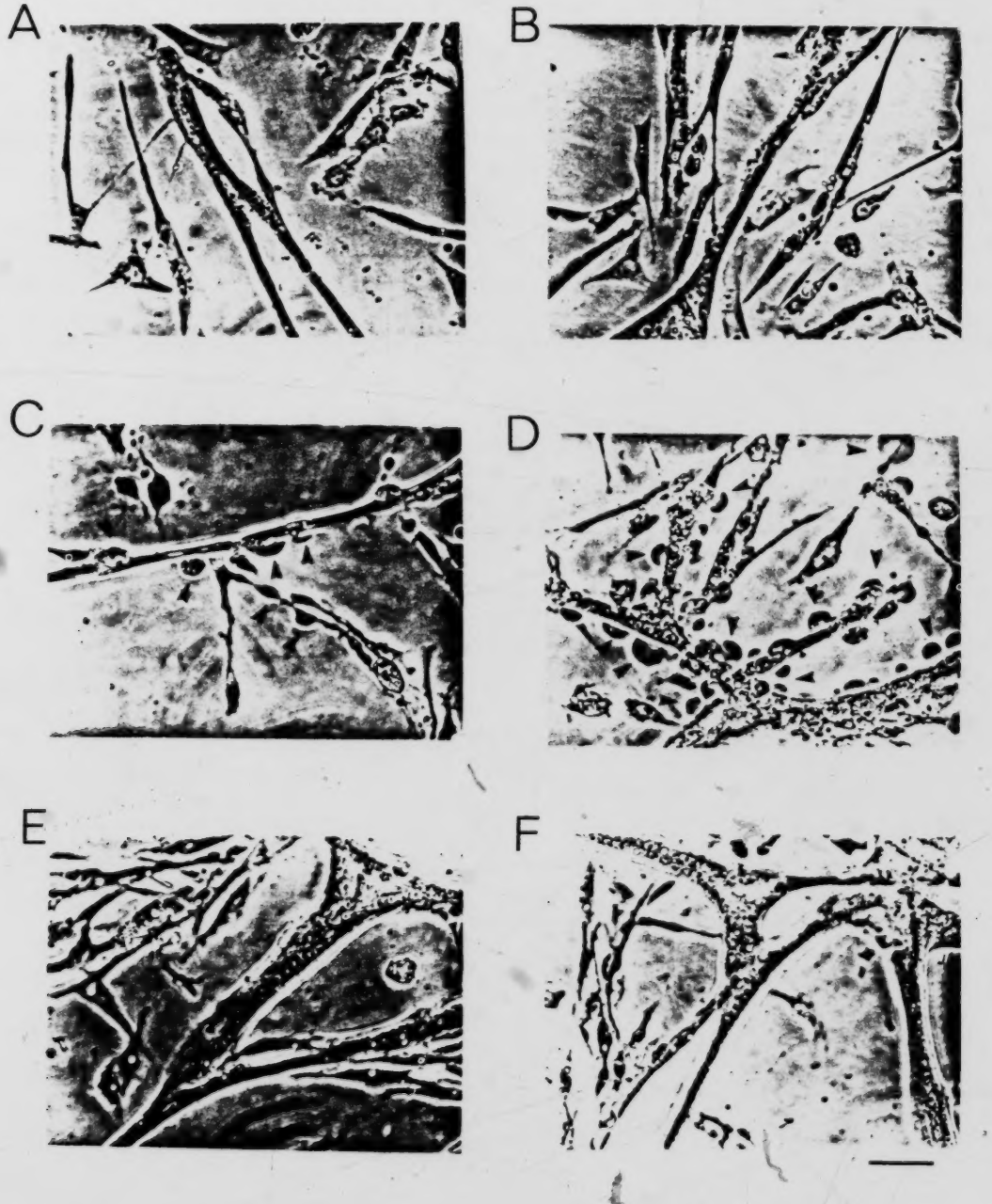
C. 3 day old fibres incubated in 5 x 10^{-5}M 9-AC for 60 minutes. Arrows (▲) indicate phase-dense blebs of cytoplasm attached to the myotubes.

D. 3 day old fibres incubated in 0.5 mM $ZnCl_2$ (Solution F) for 60 minutes. Arrows (▲) indicate phase-dense blebs of cytoplasm attached to the myotubes and rounding-up of mononuclear cells (↑).

E. 4 day old fibres incubated in 5 x 10^{-5}M 9-AC plus 10^{-6}M verapamil for 60 minutes.

F. 4 day old fibres incubated in 0.5 mM $ZnCl_2$ plus 10^{-6}M verapamil for 60 minutes.

The calibration bar represents 50 μm and applies to A-F.


A
B
C
D
E
F

zinc both increased and decreased membrane conductance in different fibres at all stages of development. The percentage of fibres in which each response was observed is presented in Table 8. In the majority of the youngest fibres the membrane conductance increased on exposure to zinc. As the fibres matured a greater proportion exhibited a reduced conductance on exposure to 0.5 mM $ZnCl_2$. The individual measurements of membrane conductance could not be averaged since the two effects would cancel out.

A few preliminary experiments were performed with zinc after removal of chloride from the medium (Figure 24). Zinc caused membrane conductance to increase significantly and membrane potential to fall significantly in older cultures (Table 9). These results indicated that zinc was not affecting solely chloride channels. Because of this and its toxicity in young fibres, no further experiments were carried out with this agent.

Figure 25 shows the effect of replacement of chloride on membrane conductance. In 3 day old fibres chloride conductance was 0.74 $mScm^{-2}$. This fell to about 0.34 $mScm^{-2}$ on days 4 and 5. Thereafter g_{Cl} fell from 0.2 $mScm^{-2}$ on day 6 to around 0.1 $mScm^{-2}$ in older cultures. Although the magnitude of the chloride conductance declined throughout the period of study, its contribution to total membrane conductance rose from 50% on days 3 and 4 to between 65 and 70% from day 5 onwards.

When 9-AC was applied to immature fibres, contrary to expectation, membrane conductance was greatly increased from a resting level of 1.1 to 3 $mScm^{-2}$ (Figure 26). However by day 5 in culture, membrane conductance was then reduced by 9-AC to the same extent as that observed with

TABLE 8

PERCENTAGE OF FIBRES RESPONDING TO 0.5 mM $ZnCl_2$

Age of Fibres Days	Increased Conductance	Decreased Conductance	No Change In Conductance
3	60	10	30
4	40	25	35
5	25	45	30
6	30	55	15
7	30	45	25
8	15	75	10
9	10	75	15
10	15	60	25

n = 20 on each day.

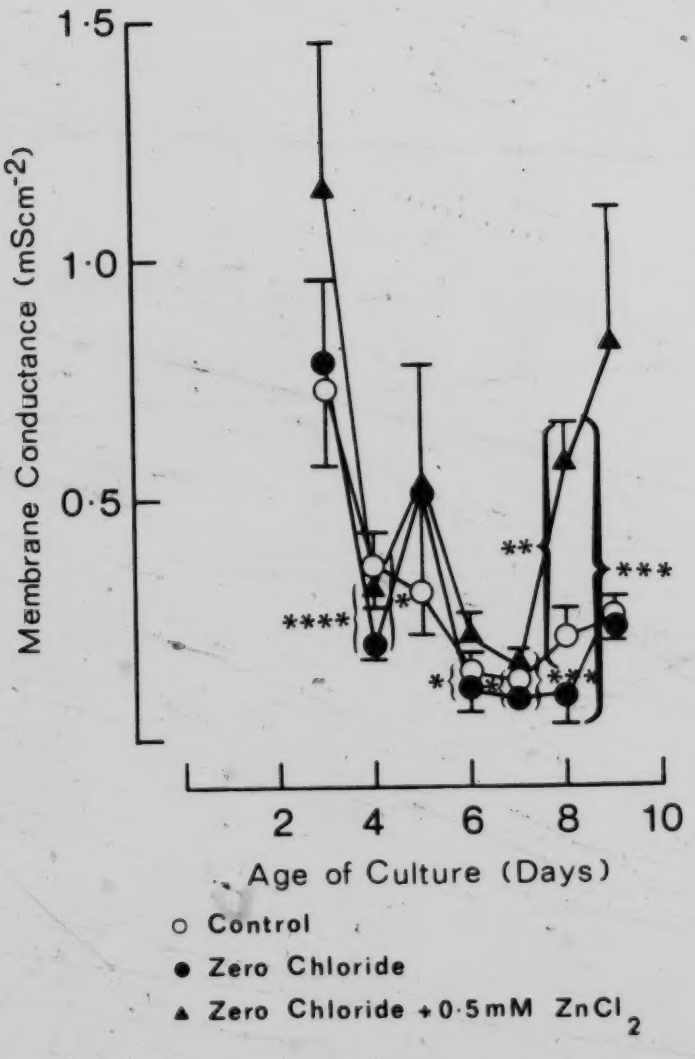
TABLE 9

EFFECT OF 0.5 mM $ZnCl_2$ ON MEMBRANE POTENTIAL OF CHICK SKELETAL MUSCLE IN CULTURE AFTER REMOVAL OF EXTERNAL CHLORIDE

Age of Culture Days	E_m in Solution E Zero Chloride mV	E_m in Solution G 0.5 mM $ZnCl_2$ mV	P
3	13.8 ± 2.7*	10.5 ± 1.0*	NS
4	29.0 ± 3.1	27.0 ± 3.8	NS
5	28.0 ± 1.15	22.6 ± 1.1	$P < 0.005$
6	35.2 ± 2.9	27.0 ± 3.5	$P < 0.05$
7	43.2 ± 1.1	35.8 ± 1.3	$P < 0.005$
8	32.8 ± 4.8	17.3 ± 4.0	$P < 0.01$
9	35.4 ± 1.7	19.7 ± 2.4	$P < 0.001$

* mean ± SEM, n = 10.

Fig. 24. Effect of removal of external chloride and then $ZnCl_2$ in the absence of chloride (Solution G) on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values were measured in the same cells 15 minutes after changeover to zero $[Cl]_o$ medium (●). The same fibres were then tested again 15 minutes after changeover to 0.5 mM $ZnCl_2$ in zero external chloride solution (▲). Each point is the mean ± SEM of 10 measurements. The test and control points were not significantly different from each other unless otherwise indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$ (Student's paired *t*-test).


1·5
1·0
0·5
Membrane Conductance (mScm-2)
2
4
6
8
10
Age of Culture (Days)
****
*
**
***
*
***
○ Control
● Zero Chloride
▲ Zero Chloride +0·5mM ZnCl2

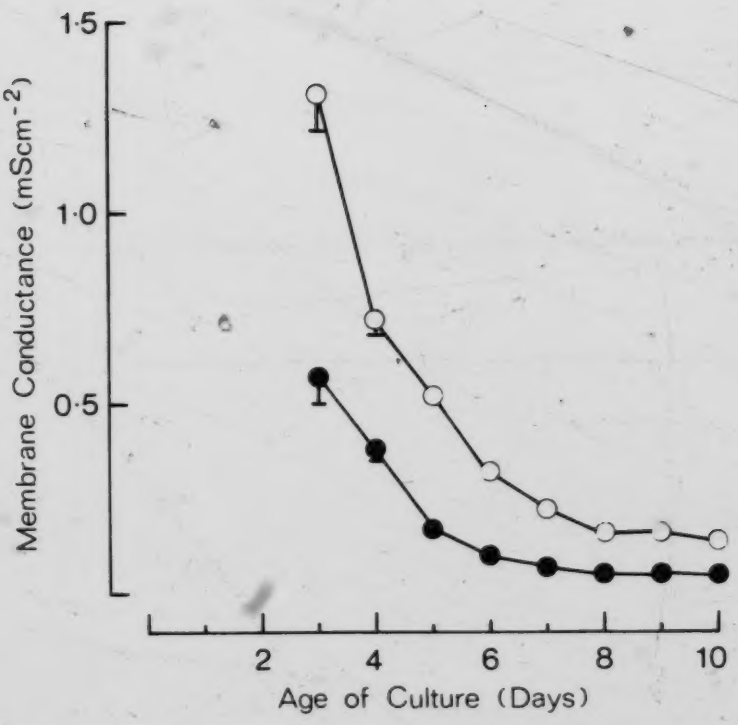


Fig. 25. Effect of removal of external chloride on G_m during development of chick muscle in culture. Control values (○) were measured in the same cells 15 minutes after changeover to zero chloride solution (●). Each point is the mean ± SEM of 30 measurements. Each test point is significantly different from the control at $P < 0.001$ using Student's paired *t*-test.

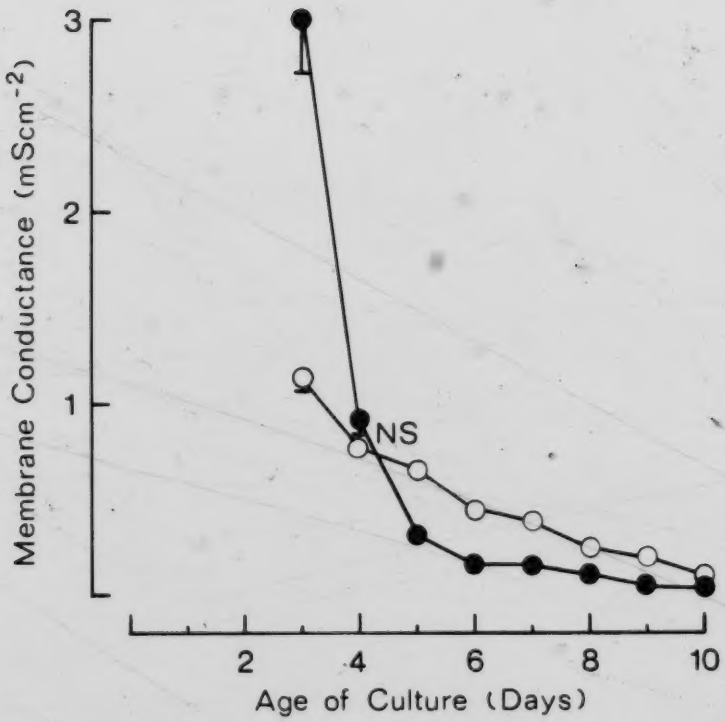


Fig. 26. Effect of 9-AC on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to 5 x 10^{-5}M 9-AC in BSS. Each point is the mean ± SEM of 30 measurements. Each test point is significantly different from the control at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated.

zero chloride (0.34 $mScm^{-2}$). Thereafter 9-AC sensitive membrane conductance fell to a final level of 0.1 $mScm^{-2}$. This component of total membrane conductance was 52% on day 5 but rose to between 60 and 70% from the sixth day of culture onwards.

If the effects of the drug on chloride conductance were removed by exposing the fibres to zero chloride and then to 9-AC in combination with zero chloride, 9-AC produced an additional effect (Figures 27 and 28). Membrane potentials were hyperpolarized back to control levels in fibres from day 5 onwards. On days 3 and 4, membrane potential was unchanged by the addition of 9-AC. Membrane conductance was significantly increased by 9-AC over levels measured in zero chloride at all stages of development (Figure 28). The increase in membrane conductance was greatest in the youngest fibres (1.3 $mScm^{-2}$). From days 4 to 6 this increase had fallen to about 0.3 $mScm^{-2}$ and thereafter the 9-AC activated conductance was about 0.15 $mScm^{-2}$.

The interaction of the potassium channel blocking drug, TEA with 9-AC was also investigated since closely related aromatic carboxylic acids had previously been found to increase potassium conductance in rat muscle (Palade and Barchi, 1977b). However in chick muscle cultures, the addition of 5×10^{-5}M 9-AC to fibres previously exposed to 10 mM TEA produced further depolarization with the exception of days 5 and 10 where E_m was not significantly affected (Figure 29). In older cultures from day 5 onwards, the addition of 5×10^{-5}M 9-AC to TEA blocked fibres produced a further 25% depression of G_m. In the 3 and 4 day old immature fibres, the addition of 9-AC increased membrane conductance after blockade of potassium channels with TEA (Figure 30).

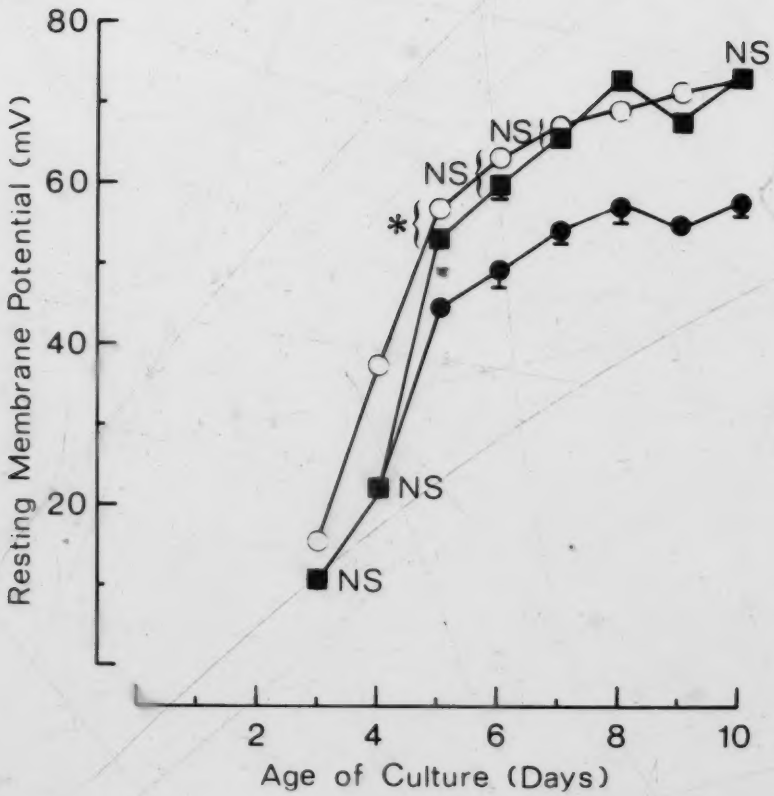
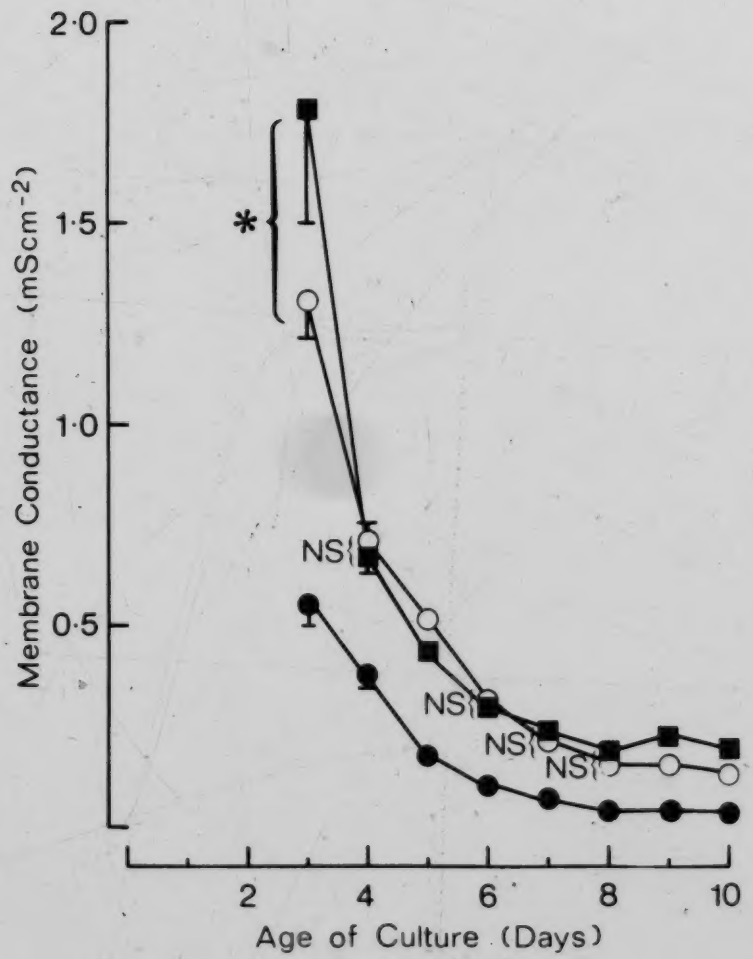


Fig. 27. Effect of removal of external chloride and then 9-AC plus zero chloride solution on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to zero $[Cl]_o$ solution (●). The same fibres were then tested again 15 minutes after changeover to zero $[Cl]_o$ plus 5×10^{-5}M 9-AC (■). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. * $P < 0.05$.

Fig. 28. Effect of removal of external chloride and then 9-AC plus zero chloride solution on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to zero $[Cl]_o$ solution. The same fibres were then tested again 15 minutes after changeover to zero $[Cl]_o$ plus 5×10^{-5}M 9-AC (■). Each point is the mean ± SEM of 30 measurements. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. * $P < 0.05$. The fibres were the same as in Fig. 27.


2·0
1·5
1·0
0·5
Membrane Conductance (mScm⁻²)
*
NS
NS
NS
NS
2
4
6
8
10
Age of Culture (Days)

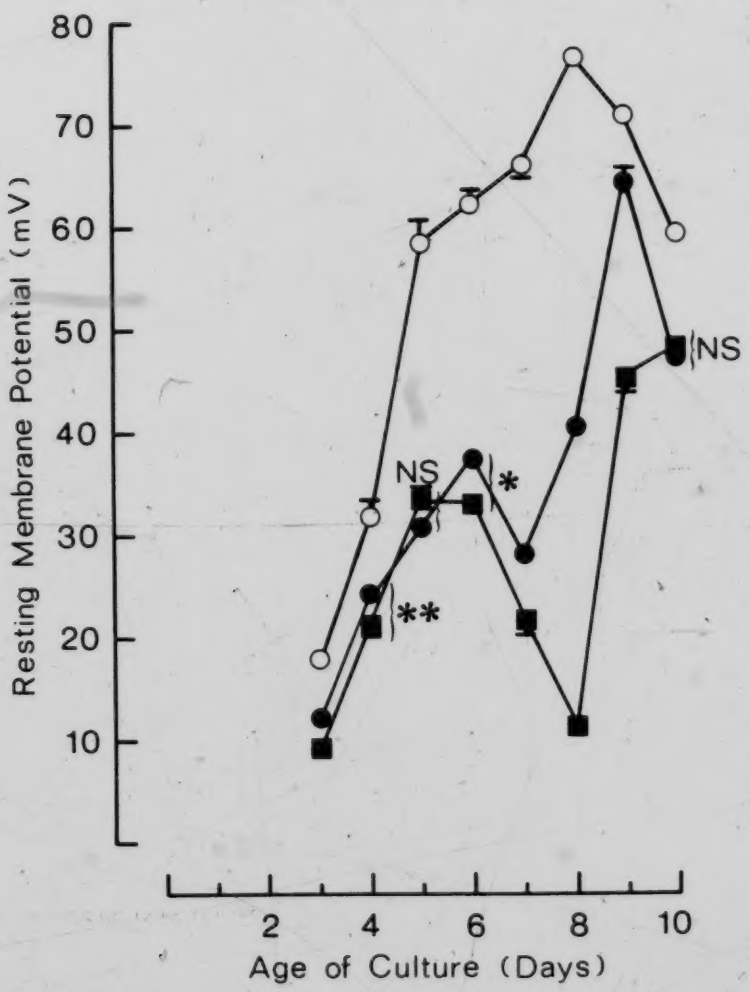
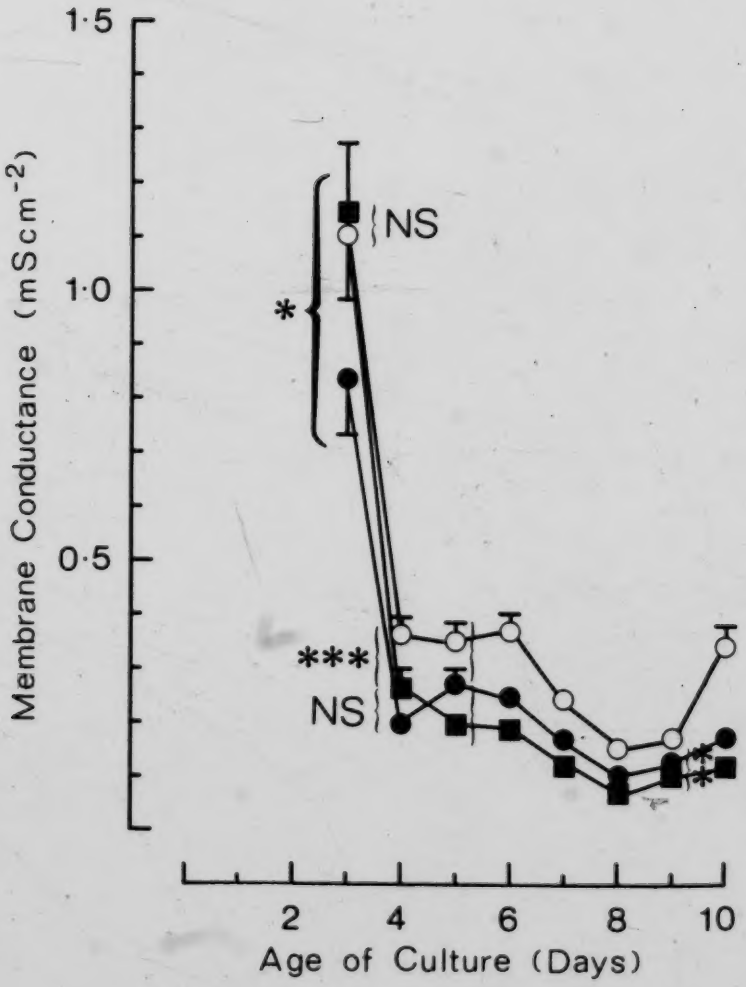


Fig. 29. Effect of TEA and then TEA plus 9-AC on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to 10 mM TEA in BSS (●). The same fibres were then tested again 15 minutes after changeover to 10 mM TEA plus 5 x 10^{-5}M 9-AC in BSS (■). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. * $P < 0.02$, ** $P < 0.01$.

Fig. 30. Effect of TEA and then TEA plus 9-AC on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values were measured in the same cells 15 minutes after changeover to 10 mM TEA in BSS (●). The same fibres were then tested again 15 minutes after changeover to 10 mM TEA plus 5 x 10^{-5}M 9-AC in BSS (■). Each point is the mean ± SEM of 30 measurements. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. * $P < 0.025$, ** $P < 0.02$, *** $P < 0.05$, { $P < 0.01$. The fibres were the same as in Fig. 29.


1·5
1·0
0·5
Membrane Conductance (mScm⁻²)
NS
*
***
NS
2
4
6
8
10
Age of Culture (Days)

C. Discussion

1. Replacement of External Chloride.

When chloride was substituted by impermeant anions, the resting membrane potential and resting membrane conductance of avian pectoral muscle were both significantly reduced throughout development in culture. Before attributing these changes solely to the loss of external chloride, possible effects on other ion conductances must be considered. The anions commonly used to replace chloride are known to reduce calcium ion activity and this may therefore affect the interpretation of results (Kenyon and Gibbons, 1977).

Methylsulphate and isethionate reduce calcium ion activity to 0.82 and 0.74 of normal respectively resulting in an effective free calcium ion concentration of 1.45 mM in Solution E (Kenyon and Gibbons, 1977; Pollard *et al.*, 1977). This reduction is not sufficient to affect membrane integrity (fibres remained intact in this solution for up to 90 minutes) and there is no evidence for a resting calcium conductance in this preparation (Chapter VII). Therefore the reduction in calcium ion activity caused by the impermeant anions is not likely to affect the resting membrane parameters investigated here and the alterations may be attributed totally to loss of inward chloride movement.

Upon replacement of chloride, the concentration gradient for this ion is reversed and chloride would tend to leave the myotubes to re-establish a Donnan equilibrium as first observed by Boyle and Conway (1941). Since the external solution was continuously changing, a new type of equilibrium would be established. Metabolic pumping of potassium would provide a retaining counterion which would reduce the outflow

of chloride. Thus chloride would no longer be passively distributed across the cell membrane in response to the potential determined by the cations, but would itself contribute to the overall membrane potential.

Viewed in this way the observed depression of membrane potential is anticipated and would persist as long as fibres were viable and able to sustain the pumped concentration of potassium. Since fibre diameter and the gross morphology of myotubes did not change after 90 minutes incubation in Solution E, this would suggest that the fibres remain viable for this period although no electrophysiological data was obtained for such fibres. Thus there was apparently no gross loss of internal ions causing osmotic crenation of the fibres and indicating the breakdown of normal homeostasis in the absence of external chloride.

Most workers are agreed that chloride on its own does not determine the resting membrane potential but that it can affect E_m transiently if its concentration gradient is altered suddenly. The original resting potential is restored as the chloride redistributes to establish a new Donnan equilibrium (Hodgkin and Horowicz, 1959b). This occurs rapidly because of the high resting conductance to the anion. However Dulhunty (1978) has recently postulated the existence of an electrogenic chloride pump in mammalian muscle which maintains the resting membrane potential more depolarized than is predicted by the Goldman-Hodgkin-Katz equation. Direct evidence for the existence of such a pump was not obtainable because the contribution from such a pump would be swamped by the large resting conductance to chloride. If a similar pump exists in avian muscle, it may contribute the depolarization observed although the major portion is probably due to the retarded outflow of chloride ions as

already discussed.

Chick pectoral muscle in primary cell culture is highly permeable to chloride although in the more immature fibres other ions must contribute significantly to resting membrane conductance since $\bar{g}Cl$ is approximately 50%. As maturation proceeds, $\bar{g}Cl$ increases to between 65 and 70% and the contribution from other ions (with the exception of potassium) must fall dramatically during development. Avian muscle in culture is therefore similar to adult skeletal muscle although $\bar{g}Cl$ constitutes a greater percentage of the resting membrane conductance in avian and mammalian fibres (Hodgkin and Horowicz, 1959b; Stanfield, 1970; Morgan *et al.*, 1975; Bryant and Camerino, 1976; Palade and Barchi, 1977a).

This contrasts with the findings of Ritchie and Fambrough (1975) and Kidokoro (1975a) for primary cultures of rat and the L6 clonal cell line. They used the observation of Hodgkin and Huxley (1959b) that the resting potential in frog muscle depolarizes suddenly when the bathing solution is changed to one containing low chloride to test whether chloride was permeable. Because neither membrane potential nor input resistance in cultures derived from mammalian muscle changed significantly, Ritchie and Fambrough, and Kidokoro each concluded that chloride was relatively impermeable. This discrepancy is not a manifestation of the culture system *per se* since the above experiment was repeated with chick muscle cultures and a transient depolarization was observed consistent with a high permeability to chloride (Dryden and Thomson, 1979).

Recent work by Engelhardt *et al.* (1976, 1977) suggests that the discrepancy between the contribution of $\bar{g}Cl$ to G_m in cultured and adult avian muscle is a tissue culture phenomenon. Engelhardt *et al.* observed

that innervation *in vitro* altered the passive membrane properties of chick muscle. Specific membrane resistance, time constant and length constant all fell after innervation: the opposite changes occur on denervation (see Table 2). These results suggest that a trophic factor from the innervating nerve is important in maintaining the electrical properties of the muscle membrane. It is possible therefore that the reduced contribution from chloride to resting membrane conductance *in vitro* may be due to the absence of this trophic factor.

This postulate is further supported by the investigation of the effect of denervation on passive membrane parameters. The increase in specific membrane resistance following denervation was originally suggested to be due to a reduction in $\bar{g}K$ (Albuquerque and Thesleff, 1968; Albuquerque and McIsaac, 1970). More recently, the component conductances have been estimated before and after denervation in mammals (Table 10). Chloride conductance fell and potassium conductance rose and when denervation changes were most marked, the proportion of G_m attributed to chloride was very similar to that found for the more mature myotubes in culture (day 5 onwards).

Additionally, a trophic effect of nerve on the development of at least one other membrane ion channel, the transient sodium channel, has been proposed on the basis of experiments using nerve extracts (Hasegawa and Kuromi, 1978; Kano *et al.*, 1979). Acetylcholine is apparently not involved (Ziskind and Harris, 1979) which also supports the hypothesis that a nerve trophic factor influences the electrophysiological maturity of the muscle membrane.

TABLE 10

EFFECT OF DENERVATION ON COMPONENT CONDUCTANCES OF MAMMALIAN SKELETAL MUSCLE

Reference	Membrane Component Conductance $\bar{g}K$ μScm^{-2}	Contribution to Total Conductance %	Membrane Component Conductance $\bar{g}Cl$ μScm^{-2}	Contribution to Total Conductance %
Bryant & Camerino, 1976. Goat gastrocnemius	175 ± 15	18.4	776 ± 49	81.6
denervated	372 ± 40	100	0	0
Camerino & Bryant, 1976. Rat EDL	260 ± 44	7.5	3196 ± 250	92.5
denervated	332 ± 38	35.8	596 ± 59	64.2
Lorković & Tomanek, 1977. Rat gastrocnemius	99	10.5	845	89.5
denervated	205	31.8	440	68.2
Lorković & Tomanek, 1977. Rat soleus	161	13.6	1025	86.4
denervated	364	65.6	191	34.4

2. Application of Zinc.

Zinc was clearly cytotoxic to the more immature myotubes causing leakage of cytoplasmic contents into the external medium followed by detachment from the substratum and cell death. This effect is probably caused by an influx of calcium ions into the fibres since raised concentrations of intracellular calcium are toxic to muscle fibres (Publicover *et al.*, 1978; Schanne *et al.*, 1979) whereas there is no such evidence for increased intracellular concentrations of other ions. This is further supported by the finding that a 500 times lower concentration of verapamil prevented the manifestations of zinc toxicity in these fibres. Verapamil is known to block currents due predominantly to calcium ions in other excitable membranes (see Reuter, 1973). The apparent absence of zinc toxicity in older myotubes may be due to the increasing maturation of the myotube. The sarcoplasmic reticulum (SR) is not well developed in immature myotubes (Ezerman, 1968) and in these fibres it may not be capable of controlling elevated calcium concentrations because the levels of Ca^{++} transport ATPase are low (Martonosi *et al.*, 1977). In older fibres, the SR is more extensive and may more readily accumulate the excess calcium and there would therefore be no evidence of membrane damage.

Invariably zinc produced depolarization but this was accompanied by either an increase or a decrease in resting membrane conductance suggesting that zinc was acting at two different sites. The percentage of fibres which exhibited depolarization accompanied by a reduction in G_m increased during maturation. This action could be explained by blockade of either potassium or chloride channels by zinc. An action on potassium channels in frog muscle was specifically excluded by Stanfield

(1970) and assuming that this finding extends to chick muscle in culture, then zinc must be blocking chloride channels in these fibres.

However this would not account for the pronounced depolarization found when fibres are exposed to zinc since chloride is normally passively distributed across the membrane according to the dictates of the major cations. One is therefore forced to postulate a metabolic source for the depolarization i.e. inhibition of Na^+-K^+ ATPase. Micromolar concentrations of zinc irreversibly inhibit this enzyme in microsomal preparations. of beef brain and eel electroplax (Hexum, 1974; Gettelfinger and Siegel, 1978). The mechanism of action was originally thought to be inhibition of the conversion of E_1-P to E_2-P which is a magnesium dependent process (Hexum, 1974). However more recent evidence suggests that some conformational restraint common to both partial reactions of the enzyme (i.e. kinase and phosphatase) is sensitive to zinc (Gettelfinger and Siegel, 1978). Inhibition of Na^+-K^+ ATPase by zinc would therefore allow an accumulation of internal sodium and concomitant loss of potassium from the myotubes resulting in depolarization.

The majority of immature fibres exhibited an increase in G_m accompanying depolarization in the presence of zinc. This response was found throughout development although to a lesser extent in older fibres. If external chloride was substituted to abolish any action on chloride channels, then the subsequent addition of zinc produced further depolarization accompanied by an increase in G_m in preliminary experiments. There are two possible candidates for this action of zinc, *viz.* sodium or calcium. Most of the evidence favours an increase in sodium conductance since Begenisich and Lynch (1974) found that internal perfusion of

zinc caused a 20-30 mV depolarization of squid axon which was reversed by the inclusion of TTX in the bathing medium. Findings discussed in subsequent chapters demonstrate that in chick muscle in culture there is no evidence of a resting calcium conductance (Chapter VII) but that there is a substantial resting sodium conductance in immature fibres which diminished during maturation (Chapter VIII). Fibres which exhibited an increase in G_m were most numerous in the earliest cultures suggesting that in these fibres zinc increases resting sodium conductance.

An alternative postulate is that zinc may increase resting membrane conductance by activating the transient channels present in the myotube membrane and maintaining them in the open state. In the immature myotubes this would allow both sodium and calcium to enter the fibres since early regenerative activity is apparently dependent on both ions (Land *et al.*, 1973) and in the more mature fibres the calcium component is lost. The reversal of the action of zinc by TTX in squid axon would support this view. However on the basis of the preliminary experiments reported here, it is impossible to resolve the two mechanisms. The incontrovertible conclusion remains that the lack of specificity of action makes zinc an unsatisfactory antagonist of chloride conductance in developing myotubes.

3. Application of Anthracene-9-carboxylic Acid.

Anthracene-9-carboxylic acid exhibited cytotoxicity similar to although less severe than that observed with zinc and which was also prevented by the addition of verapamil. Presumably this action is associated with an increased influx of calcium although the mechanism by which it is brought about may not be the same for the two agents. 9-AC produced different

effects on E_m and G_m depending on the age of the myotubes under investigation and on whether chloride was or was not present in the bathing fluid. Discussion of the actions of 9-AC in immature fibres is delayed until later (Chapter IX) and only the effects on myotubes of 5 days and older are considered here.

The application of 9-AC in BSS to these fibres produced a reduction in G_m which was similar to that found upon replacement of external chloride by impermeant anions (Figures 25 and 26). This was accompanied by a depolarization which was progressively reduced on successive days until by the tenth day of culture no depolarization was evident. Blockade either of potassium or chloride channels is possible to account for the reduction in G_m. The former mechanism was specifically excluded since the addition of 9-AC to fibres blocked maximally by TEA produced a further reduction in both E_m and G_m. Therefore in chick pectoral muscle, 9-AC blocks only chloride channels from the fifth day of culture onwards.

The progressive reduction in the depolarization on consecutive days caused by 9-AC is not readily explicable although it was a consistent feature of the drug's action. It can also be observed in the experiments with TEA (Figure 29). The most mature myotubes resemble adult mammalian muscle since a large reduction in resting membrane conductance with no accompanying alteration in E_m has been reported for 9-AC (Bryant and Morales-Aguilera, 1971; Palade and Barchi, 1977b). If, in immature myotubes, chloride assists in determining resting membrane potential possibly by providing a counterion for the major cations then blockade of free chloride movement by 9-AC would produce depolarization. As the myotubes mature and the P_{Na}/P_K ratio falls, then the membrane potential would become less

dependent on chloride and more dependent on the distribution of cations. 9-AC would therefore cause less and less depolarization as myogenesis proceeded.

In the absence of chloride ions, 9-AC causes the opposite response to that described above i.e. hyperpolarization and an increase in resting membrane conductance. This action can only be attributed to an increase in potassium efflux from the fibres. This may occur through the normal potassium channel since closely related aromatic carboxylic acids increase resting potassium conductance in goat and rat muscle (Bryant and Morales-Aguilera, 1971; Palade and Barchi, 1977b). If this were the case, then 9-AC would be expected to produce a lesser effect on G_m in the presence of chloride than in its absence, which evidently did not occur (Figures 26 and 28). However if potassium was effluxing through the chloride channel then this would account for the observed result. Normally potassium would not exit by this route because of mutual repulsion between the ions and fixed positive charges postulated to line the chloride channel. This would no longer apply if 9-AC were bound to these sites thereby neutralizing them. Potassium could then leave the myotubes under the influence of its concentration gradient by this route. This effect would not occur in normal medium because of the competition between 9-AC and chloride ions for these hypothetical binding sites. Anthracene-9-carboxylic acid is a more specific antagonist of chloride conductance in developing myotubes than zinc and produces similar estimates of $\bar{g}Cl$ to those obtained by removal of chloride from the bathing solution. However it is not completely specific, especially in the more immature myotubes, and should therefore be used solely for confirmation of replacement experiments.

VII. EFFECTS OF MULTIVALENT CATIONS REPUTED TO AFFECT CALCIUM CONDUCTANCE

A. Introduction

There is no evidence that adult excitable membranes maintain a resting conductance to calcium but its influx is increased during activity (Hodgkin and Keynes, 1957; Fatt and Ginsborg, 1958; Bianchi and Shanes, 1959; Hagiwara and Naka, 1964; Baker *et al.*, 1971a, 1971b; Stanfield, 1977). In muscle cultures, calcium dependent regenerative activity has also been reported (Land *et al.*, 1973; Kidokoro, 1973, 1975a, 1975b; Kano and Shimada, 1973; Kano and Yamamoto, 1977). Work reported in the previous chapters reveals that, in immature chick muscle in tissue culture, there is an initial large resting membrane conductance which is only partially attributable to potassium and chloride. The remaining unidentified conductance (about 0.35 $mScm^{-2}$ on day 3 declining to 0.2 and 0.05 $mScm^{-2}$ on days 4 and 5 respectively) apparently disappears over a 2 to 3 day period in culture since in cultures from day 6 onwards the contributions from potassium and chloride account for the total resting membrane conductance.

To investigate the nature of this conductance, experiments were performed using three multivalent cations which block calcium currents in excitable membranes; *viz.* manganese, cobalt and lanthanum (Reuter, 1973). Measurement of membrane conductance in the absence of external calcium was impossible due to irreversible membrane damage following penetration by the recording electrode (Dryden, Fambrough, personal communications). External calcium was not removed since it is essential for the maintenance of membrane integrity as a counter ion for the surface negative charges on the membrane (Gilbert and Ehrenstein, 1969).

B. Results

The polyvalent cations were added to the BSS to a final concentration of 2 mM in the presence of 1.8 mM calcium. This concentration was sufficient to abolish slow regenerative potentials generated by applying 100 msec depolarizing pulses to 3 or 4 day old fibres which had been hyperpolarized to a holding potential of -80 mV by the passage of a steady inward current. These slow regenerative potentials in immature cultured muscle are due to inward calcium movement (Kidokoro, 1973; 1975a; 1975b; Kano and Shimada, 1973; Fukuda, 1974).

Figures 31 and 32 illustrate the effect of 2 mM $CoCl_2$ on membrane potential (E_m) and conductance during myogenesis. Cobalt depressed E_m at all stages of development but to a lesser extent in young fibres (days 3 to 5). The sequence of development of G_m proceeded as described previously (Chapter IV) and was unaffected by the divalent cation (Figure 32). Similar data was obtained when 2 mM manganese was used (Figures 33 and 34) although a marked hyperpolarization was observed on day 4. Membrane conductance was reduced by 0.4 $mScm^{-2}$ when manganese was applied on day 3 but this was not significantly different from the control ($P > 0.05$).

Figures 35 and 36 show the action of the trivalent cation, lanthanum, on E_m and G_m which did not produce the same results as the other two ions. Membrane potential was depressed at all stages of development although on day 4 the depolarization was not significant. When membrane conductance was calculated, it was found that G_m increased in the presence of 2 mM lanthanum in contrast to the divalent cations. The increase was most marked in the immature fibres (day 3 - 1.7 $mScm^{-2}$, day 4 - 0.33 $mScm^{-2}$, day 5 - 0.12 $mScm^{-2}$) but then declined to an increase of around 0.06 $mScm^{-2}$ in the older fibres (days 6 *et seq.*).

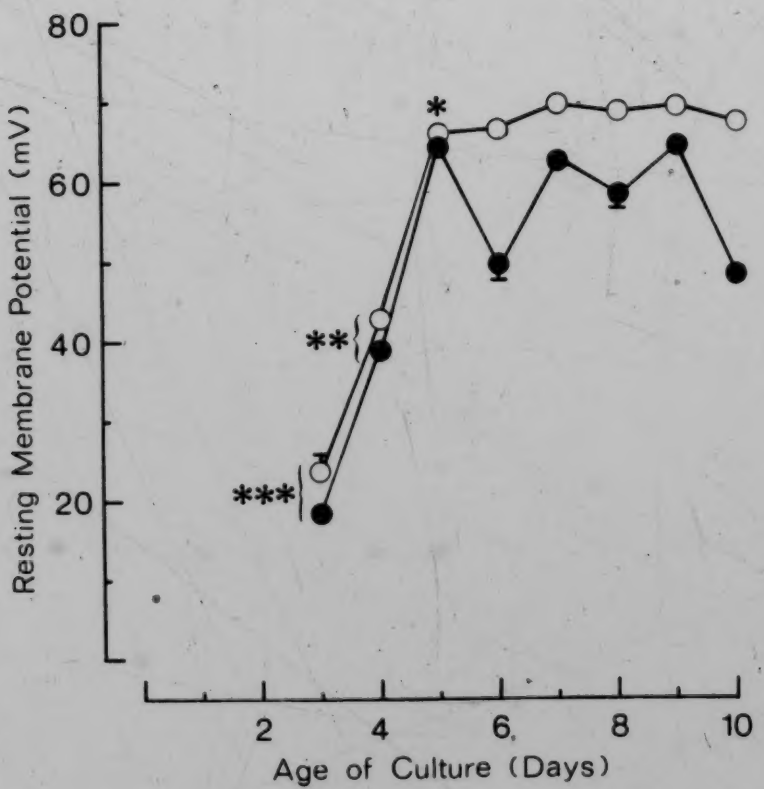


Fig. 31. Effect of cobalt on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to 2 mM Co medium (●). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. * $P < 0.02$; ** $P < 0.01$; *** $P < 0.005$.

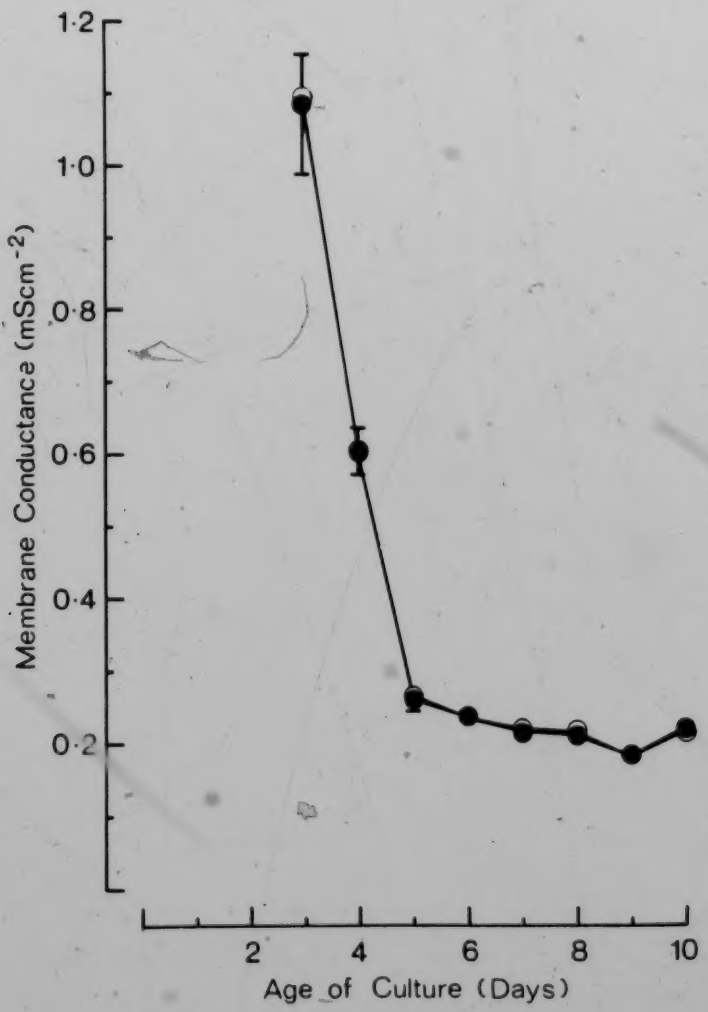


Fig. 32. Effect of cobalt on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to 2 mM Co medium. Each point is the mean ± SEM of 30 measurements. All points are non-significant ($P > 0.05$, Student's paired t-test). The fibres were the same as in Fig. 31.

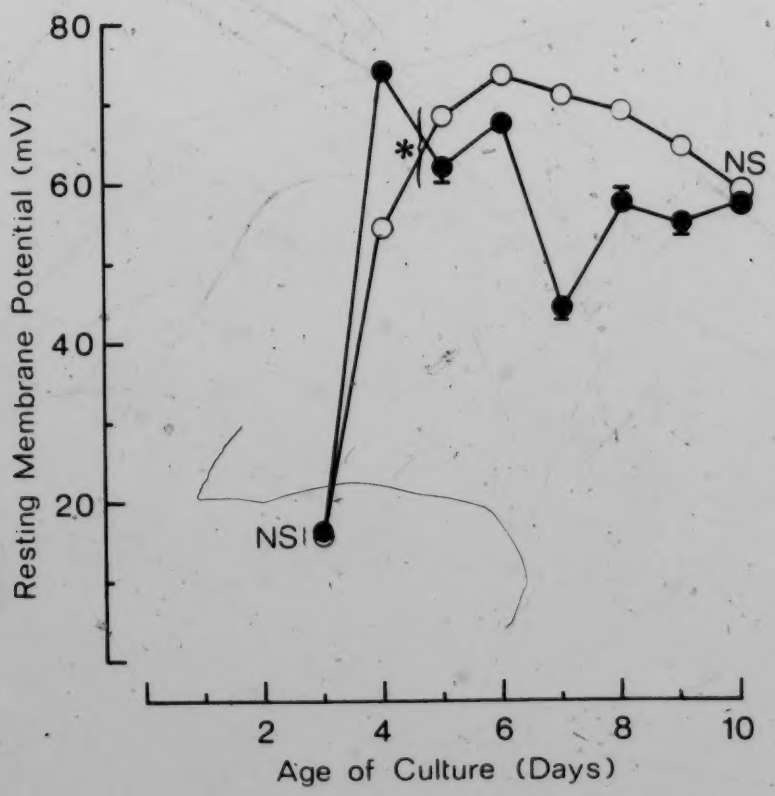


Fig. 33. Effect of manganese on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to 2 mM Mn medium (●). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. * $P < 0.005$.

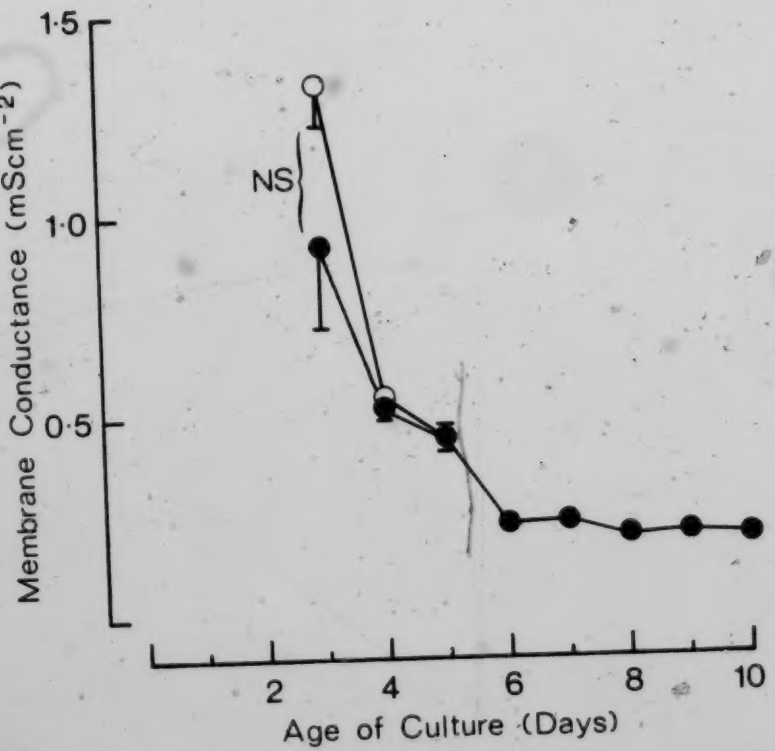


Fig. 34. Effect of manganese on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to 2 mM Mn medium. Each point is the mean ± SEM of 30 measurements. All points are non-significant ($P > 0.05$, Student's paired *t*-test). The fibres were the same as in Fig. 33.

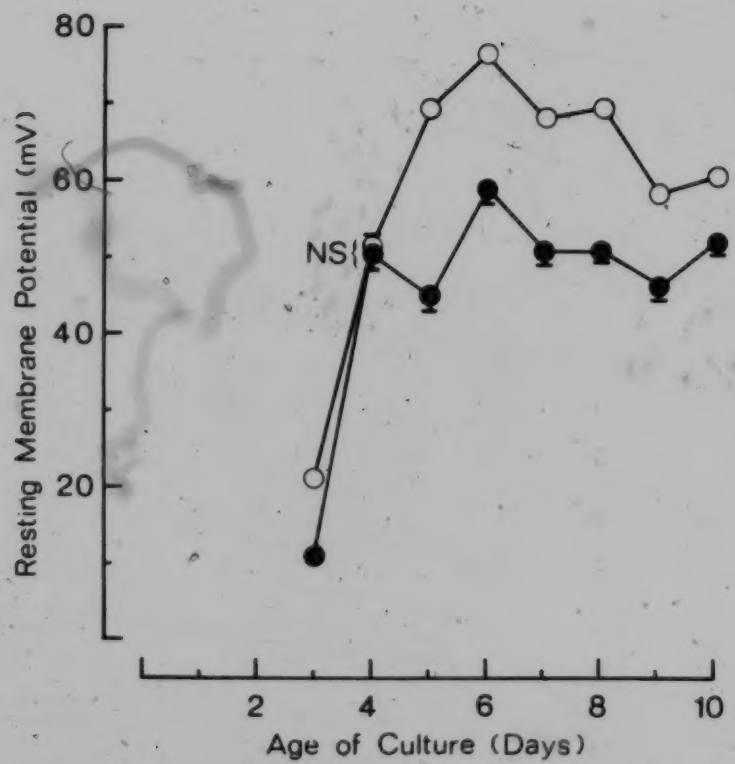


Fig. 35. Effect of lanthanum on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to 2 mM La medium (●). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control at $P < 0.001$ (Student's paired t-test) unless otherwise indicated.

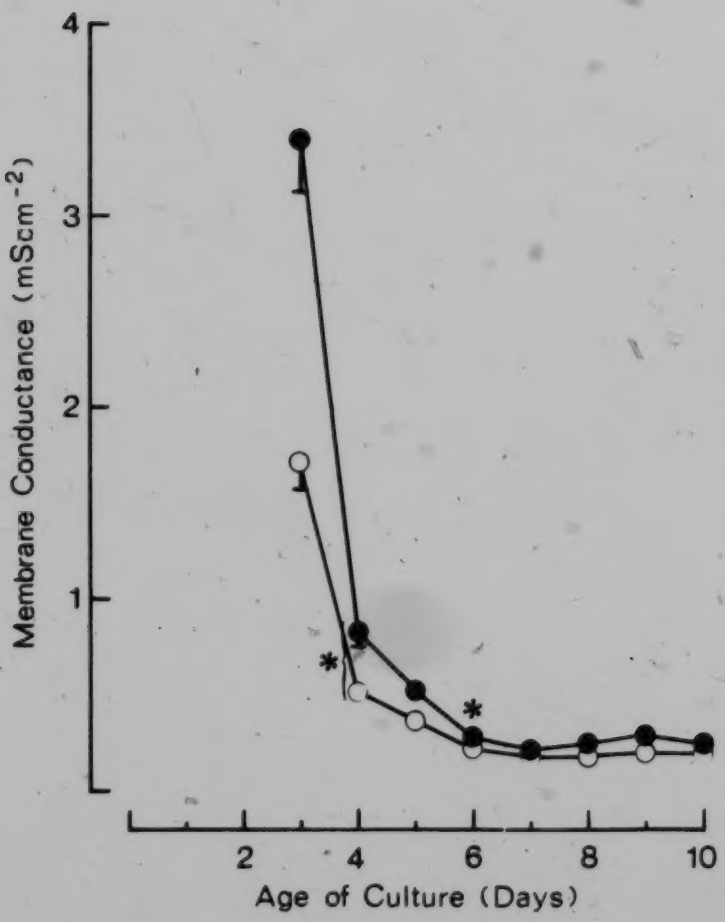


Fig. 36. Effect of lanthanum on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to 2 mM La medium. Each point is the mean ± SEM of 30 measurements. Each test point is significantly different from the control at $P < 0.001$ unless otherwise indicated (Student's paired *t*-test). The fibres were the same as in Fig. 35. * $P < 0.005$.

C. Discussion

The three multivalent cations caused depolarization throughout development of chick muscle in culture although on one occasion manganese caused hyperpolarization. Neither divalent cation affected resting membrane conductance as opposed to lanthanum which produced an increase in G_m throughout development. This increase was greater in the more immature myotubes. Because most investigators have employed these agents solely to suppress regenerative potentials there is little comparable data for resting membrane parameters (Reuter, 1973; Land *et al.*, 1973; Kano and Shimada, 1973; Fukuda, 1974).

Since the depolarization caused by manganese and cobalt was unaccompanied by a concomitant alteration in G_m, this suggests that the reduction in G_m is not mediated by membrane ion channels and alternative mechanisms must be sought. One such proposal is the displacement of membrane bound calcium ions leading to an increase in intracellular calcium levels (Stefani and Uchitel, 1976; Terakawa *et al.*, 1977). Elevation of internal calcium may produce depolarization in immature fibres where the SR is not well developed (Ezerman, 1968) but in the more mature fibres the excess calcium would be taken up by the SR. An increase in $[Ca]_i$ would be evident either as contracture or membrane rupture (Chapter VI). However membrane integrity was unaffected by either manganese or cobalt.

A more viable alternative is inhibition of Na^+-K^+ ATPase. Both ions produced 20% enzyme inhibition at 200 μM by competing with magnesium (Prakash *et al.*, 1973). Since ten times higher concentrations of both ions were used, then inhibition of Na^+-K^+ ATPase may account for the observed depolarization. This action of cobalt and manganese could be a

reflection of the relative immaturity of the myotube membrane since no depolarization has been reported in adult excitable membranes i.e. frog and barnacle muscle, squid axon, or Purkinje fibres (Weakly, 1973; Chiarandini and Stefani, 1973; Hagiwara and Nakajima, 1966; Terekawa *et al.*, 1977; Vitek and Trautwein, 1971). The Na^+-K^+ ATPase in chick myotubes in culture may differ from that found in adult tissue by being either more susceptible or accessible to the action of manganese and cobalt. There is general agreement however that the divalent cation antagonists of calcium do not affect the resting membrane conductance of excitable membranes. This is consistent with the present observations and indicates that there is no resting calcium conductance present during myogenesis of chick pectoral muscle *in vitro*.

Lanthanum differs from the two divalent cations since it causes depolarization accompanied by an increase in resting membrane conductance. Being a trivalent cation, it is more strongly electropositive than either manganese or cobalt. This difference has been suggested to account for the increased potency of lanthanum as a channel blocking agent (Weiss, 1974) and as a screening agent of membrane surface charge (Hagiwara and Takahashi, 1967) i.e. the 'supercalcium' of Takata *et al.* (1966). Lanthanum stimulates the spontaneous release of biogenic amines from nerve terminals and secretory cells (Weiss, 1974). This has been postulated to be caused by the displacement of membrane bound calcium from superficial binding sites. Such a mechanism has been demonstrated in adult and tissue cultured heart muscle (Sanborn and Langer, 1970; Langer and Frank, 1972). If lanthanum is similarly displacing calcium from chick myotube membranes, then the disruption of the membrane architecture may be pro-

ducing a non-specific increase in membrane conductance and a concomitant depolarization rather than an alteration in the conductance of one specific ion channel.

An additional component of the depolarization may come from inhibition of Na^+-K^+ ATPase since lanthanum has been reported to cause maximal inhibition of the enzyme at 100 μM in an adult heart preparation (Naylor and Harris, 1976). Depolarization caused by lanthanum has been observed in crayfish giant axons (Hartz and Ulbricht, 1973) and guinea pig papillary muscle (Ravens, 1975) although it is not a general feature. No depolarization of L6 clone muscle was observed upon application of millimolar concentrations of lanthanum and membrane conductance was reduced (Kidokoro, 1975b). The discrepancy between these and the present results may be due to species difference or to the electrophysiological differences between the clonal line and primary cultures (see Chapter I, Section B). An electrophysiological difference rather than a species difference is indicated by the observation that lanthanum caused significant depolarization of primary cultures of rat cardiac muscle (Kitzes and Berns, 1979).

In smooth, cardiac and skeletal muscle the movement of ^{45}Ca is reduced or prevented by the three multivalent cations employed in these experiments (Van Breemen, 1969; Godfraind, 1976; Langer *et al.*, 1975; Langer and Frank, 1972). It would be expected therefore that if there was a significant contribution from calcium to resting membrane conductance in chick skeletal muscle during myogenesis then that portion of G_m should be reduced by the application of one of the multivalent ions. However, it appears that calcium does not contribute to the resting

membrane conductance during the differentiation of chick muscle in culture and therefore that it does not contribute to the unidentified conductance present in immature fibres.

VIII. EFFECTS OF AGENTS REPUTED TO AFFECT SODIUM CONDUCTANCE

A. Introduction

In adult skeletal muscle, the presence of a resting sodium conductance was not considered until 1949 (Hodgkin and Katz), although the importance of potassium in the determination of resting membrane potential had been recognized since the turn of the century (Bernstein, 1902; 1912). The membrane permeability to sodium of frog muscle was calculated to be 0.013 that to potassium (Hodgkin and Horowicz, 1959a). This was later extended to nerve based on the observation that the application of TTX to resting squid axon produced a small hyperpolarization (Freeman, 1969; Narahashi, 1972).

Several workers have investigated the resting permeability to sodium of muscle in tissue culture. Dryden *et al.* (1974) obtained an estimate of P_{Na}/P_K in chick myoblasts of 0.94 which subsequently declined to 0.43 in 5 day myotubes. This indicated that the fibres became relatively less permeable to sodium during myogenesis. A similar reduction in P_{Na}/P_K has been reported for primary cultures of rat muscle (Ritchie and Fambrough, 1975). In sodium replacement experiments, Kidokoro (1975a) found that the resting membrane potential of L6 clone myoblasts and myotubes was unaffected. However in primary cultures of chick muscle, depolarization was observed when sucrose was used to replace sodium and chloride (Dryden *et al.*, 1974). Myoblasts and early myotubes were little affected, but the depolarization increased progressively as the fibres matured. Since both Na and Cl were replaced in this study the two reports are not directly comparable but are not incompatible.

To investigate whether there is a contribution from sodium to

resting membrane conductance in primary chick muscle cultures, a sodium-free solution was prepared (Solution D). Results obtained using this solution were compared with the unidentified conductance present in immature fibres which could not be attributed to any of the other major ions. Experiments performed with verapamil are reported here since verapamil produced a similar reduction of resting membrane conductance to that observed with solution D. Although verapamil is known to block slow current channels in excitable membranes (Reuter, 1973) its analogue, D600 (methoxyverapamil) has recently been shown to affect the transient sodium channels in heart cell cultures (Galper and Catterall, 1978). The effect of TTX on the resting membrane parameters of chick muscle in culture was also investigated since it blocks resting sodium conductance in squid axon (Freeman, 1969; Narahashi, 1972).

Finally in order to characterize further the development of the transient sodium channel in cultured muscle, experiments were performed with two agents known to alter the kinetics of channel gating in adult membranes. These are veratridine and condylactis toxin (CTX). In Hodgkin-Huxley terms, veratridine opens the transient channels of nerve and skeletal muscle (i.e. opens the m^3 gate) and then maintains them in the activated state by preventing the inactivation mechanism (h gate) from closing the channels (Shanes, 1958; Ulbricht and Flacke, 1965); Catterall and Nirenberg, 1973). CTX on the other hand requires prior activation of the transient sodium channels (in lobster axon) before it can then act to prevent or markedly slow inactivation (Narahashi *et al.*, 1969).

B. Results

1. Resting Membrane Conductance.

In these experiments, replacement of sodium by sucrose was not possible since sucrose is a non-electrolyte and therefore cannot carry current during estimation of input resistance. Furthermore ion replacement by a non-electrolyte necessitates replacement of the counter ion i.e. chloride which would complicate the analysis of results. Therefore choline was used to replace sodium. To prevent choline from inactivating the acetylcholine receptors, 10^{-3}M d-tubocurarine was included in the choline chloride solution (Solution D).

Figures 37 and 38 illustrate the lack of effect of 10^{-3}M d-tubocurarine alone on resting membrane potential and resting membrane conductance. The next two figures show the effect of replacement of external sodium by choline on both parameters. Membrane potential was depressed at all stages of development but membrane conductance was reduced only on the first three days of the experiment i.e. days 3 to 5 inclusive (Figures 39 and 40). Thereafter membrane conductance was unaffected by removal of external sodium. The magnitude of the conductance which was blocked by the choline chloride solution was 0.43 $mScm^{-2}$ on day 3, 0.13 $mScm^{-2}$ on day 4; and 0.05 $mScm^{-2}$ on day 5.

If 10^{-7}M tetrodotoxin was included in the BSS, no significant alteration in either membrane potential or resting membrane conductance was found throughout development (Figures 41 and 42).

Verapamil, at a concentration of 5×10^{-6}M, (McCall, 1976) caused depolarization on all days except day 4 and 10 when membrane potential was not significantly affected by exposure to the drug (Figure 43).

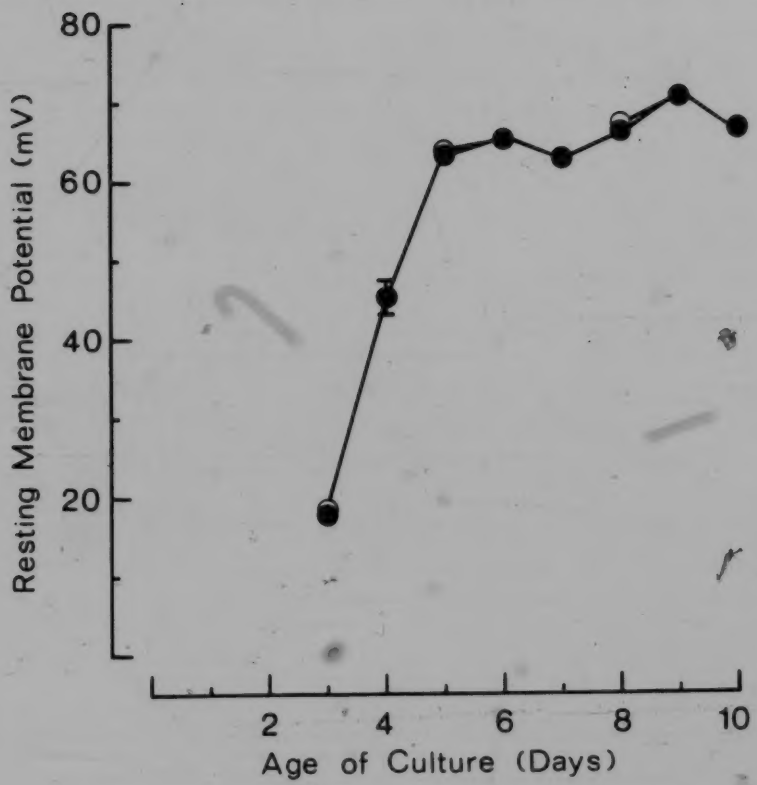


Fig. 37. Effect of d-tubocurarine on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to BSS containing 10^{-3}M d-tubocurarine (●). Each point is the mean ± SEM of 30 observations. Test measurements were not significantly different from the control (Student's paired *t*-test).

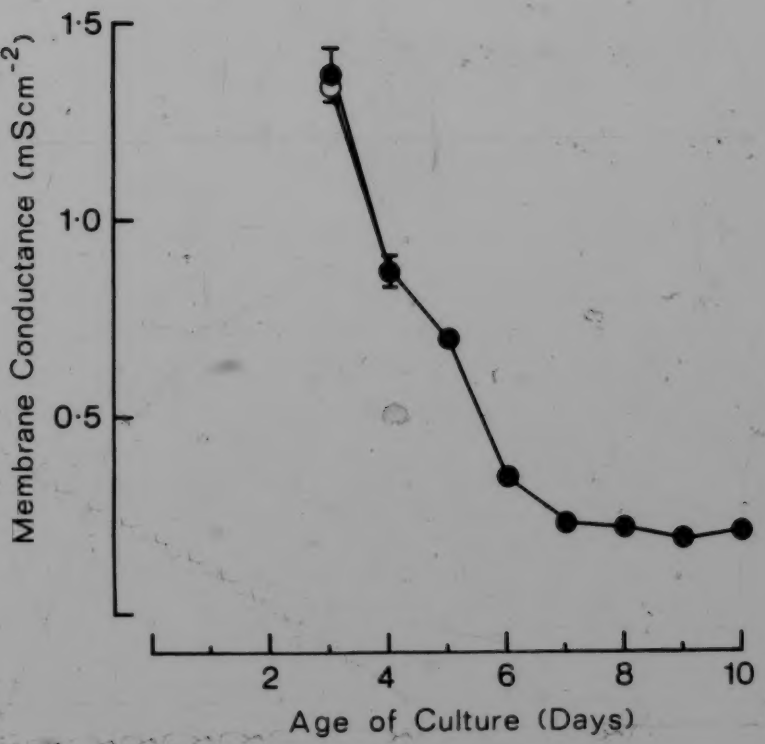


Fig. 38. Effect of d-tubocurarine on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to BSS containing 10^{-3}M d-tubocurarine. Each point is the mean ± SEM of 30 measurements. Test points were not significantly different from the control (Student's paired *t*-test). The fibres were the same as in Fig. 37.

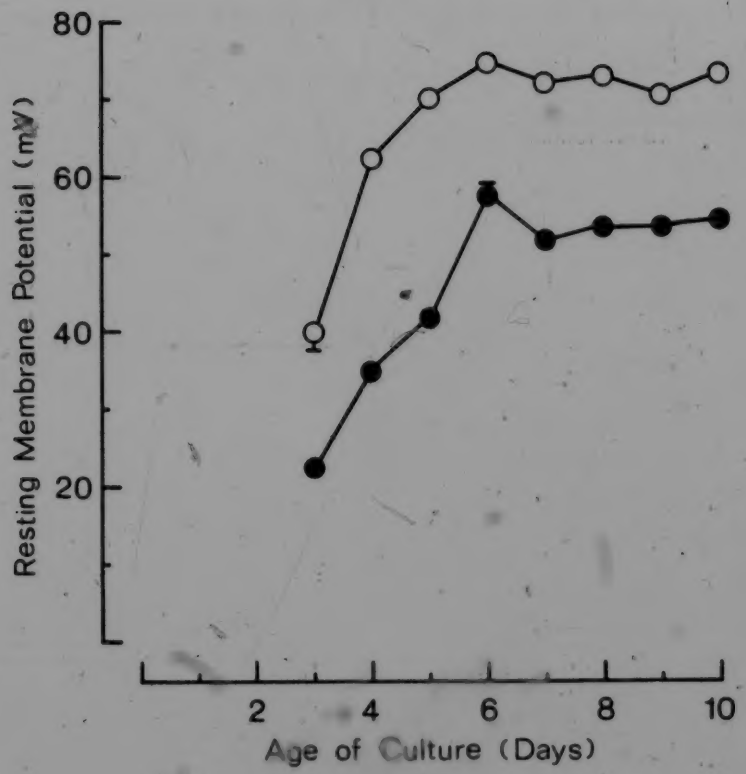


Fig. 39. Effect of choline chloride plus 10^{-3}M d-TC on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to choline chloride plus 10^{-3}M d-TC (Solution D) (●). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control at $P < 0.001$ using Student's paired *t*-test.

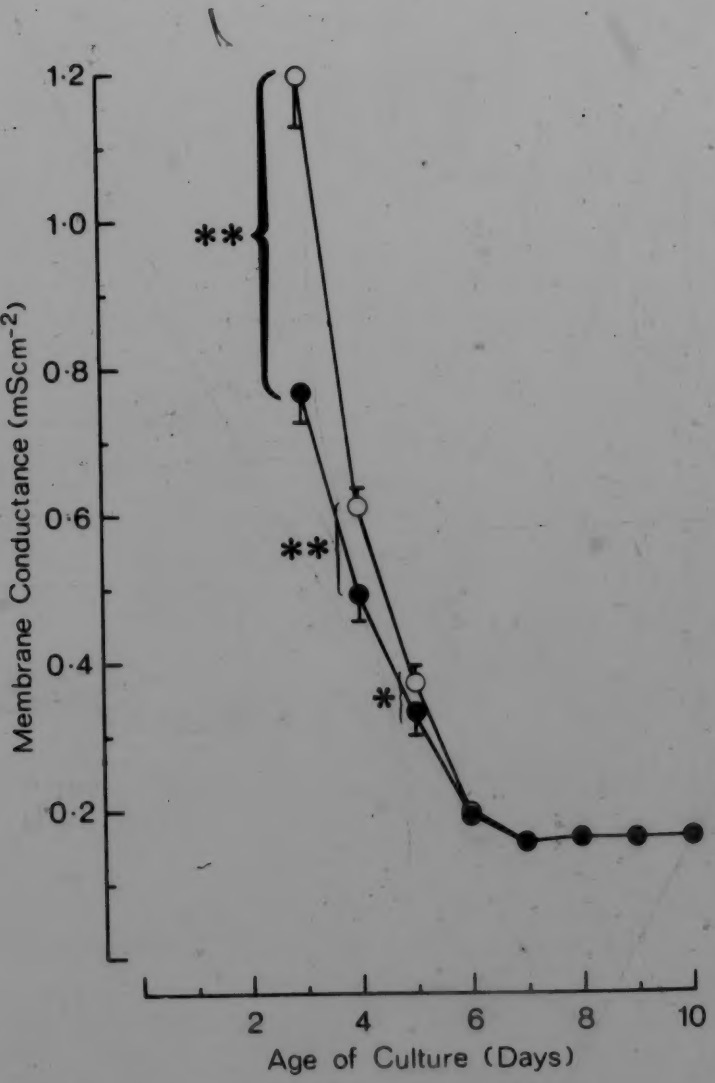


Fig. 40. Effect of choline chloride plus 10^{-3}M d-TC on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to choline chloride plus 10^{-3}M d-TC (Solution D). Each point is the mean ± SEM of 30 measurements. Test points were not significantly different from the control (Student's paired t-test) unless otherwise indicated. * $P < 0.005$, ** $P < 0.001$. The fibres were the same as in Fig. 39.

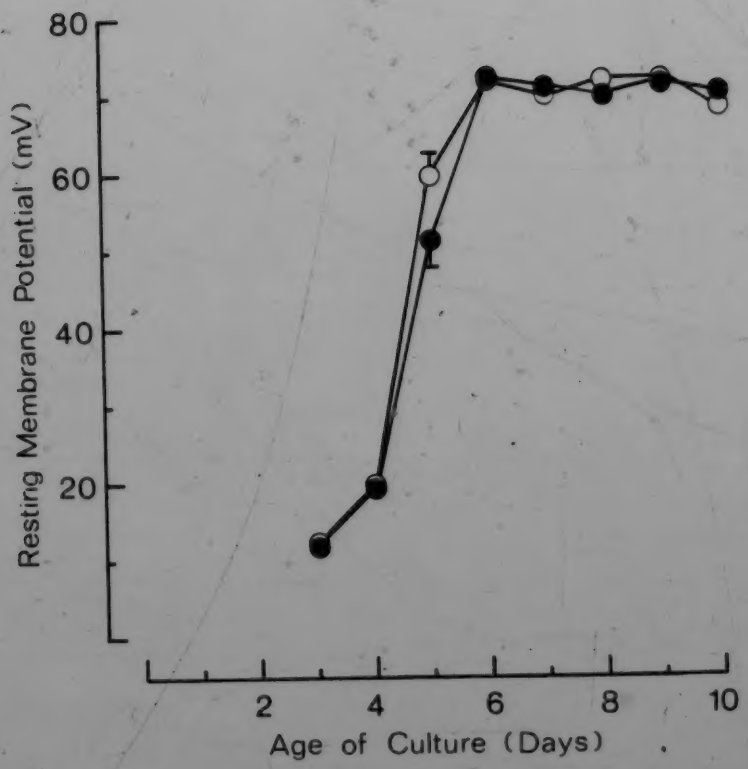


Fig. 41. Effect of TTX on E_m during development of chick skeletal muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to 10^{-7}M TTX in BSS (●). Each point is the mean ± SEM of 30 observations. Test points were not significantly different from the control (Student's paired *t*-test).

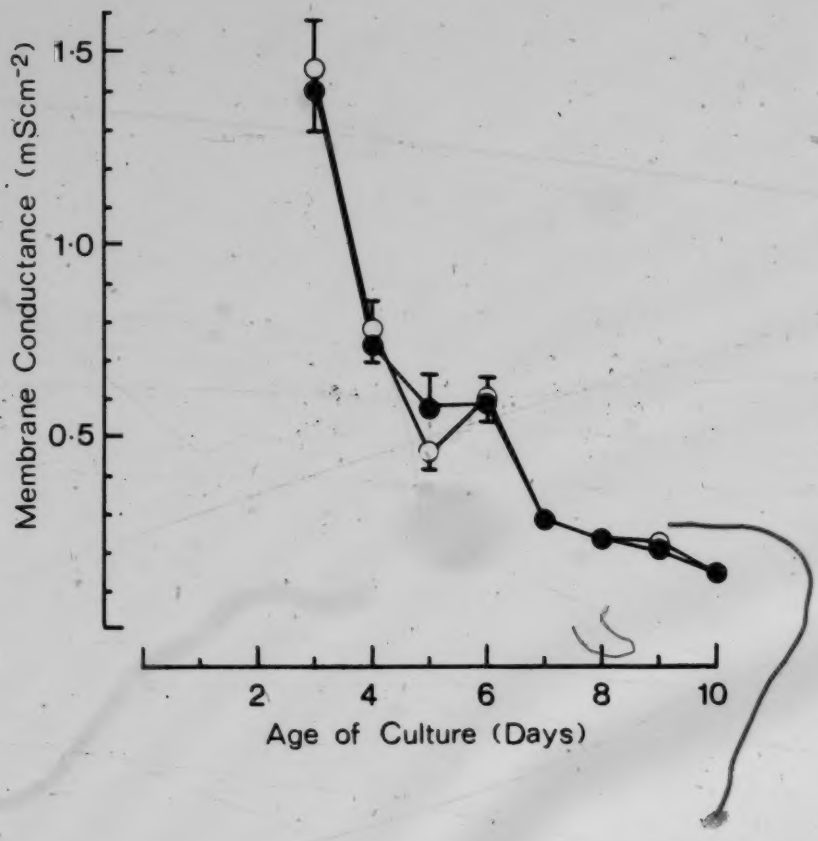


Fig. 42. Effect of TTX on G_m during development of chick skeletal muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to 10^{-7}M TTX in BSS. Each point is the mean ± SEM of 30 measurements. Test points were not significantly different from the control using Student's paired *t*-test. The fibres were the same as in Fig. 41.

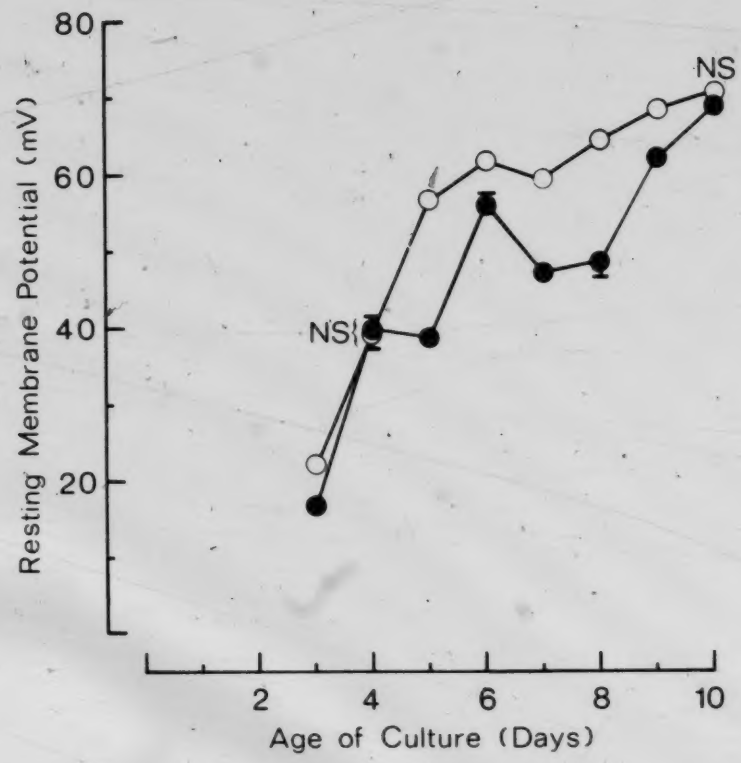


Fig. 43. Effect of verapamil on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to 5 x 10^{-6}M verapamil HCl in BSS (●). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated.

However membrane conductance was reduced by 0.35 $mScm^{-2}$ on day 3 and by 0.17 $mScm^{-2}$ on day 4 (Figure 44). A non-significant reduction of 0.01 $mScm^{-2}$ was observed on day 5 but thereafter the addition of verapamil did not alter resting membrane conductance. This effect was similar both in magnitude and duration to that seen when external sodium was replaced by choline. Accordingly, the effect of verapamil on immature myotubes was examined after external sodium was replaced by choline. Membrane potential was unaffected by verapamil on day 3 (Figure 45) and on days 4 and 5 a small 5 mV depolarization or hyperpolarization occurred. On day 6, there was a 7 mV depolarization when verapamil was added to the choline chloride solution. After resting membrane conductance had been reduced by the sodium free solution the addition of $5 \times 10^{-6}M$ verapamil produced no further alteration in resting membrane conductance (Figure 46). By day 6, removal of external sodium and verapamil alone did not affect membrane conductance, so the combination of the two was expected to have no effect on membrane conductance as indeed occurred.

2. Transient Sodium Channel.

$10^{-5}M$ veratridine hydrochloride caused 5-10 mV depolarization in young fibres up to the fifth day of culture (Figure 47). Thereafter a marked 30 mV depolarization was observed upon addition of the drug. In young fibres, membrane conductance was unaffected by the addition of veratridine. However from day 6 onwards, a marked increase in membrane conductance occurred on the addition of veratridine of about 0.5 $mScm^{-2}$ with a peak increase on day 6 of 1.1 $mScm^{-2}$ (Figure 48).

Condylactis toxin (CTX) was applied to individual fibres by microperfusion of a 0.2 mg/ml solution in BSS. In control experiments BSS

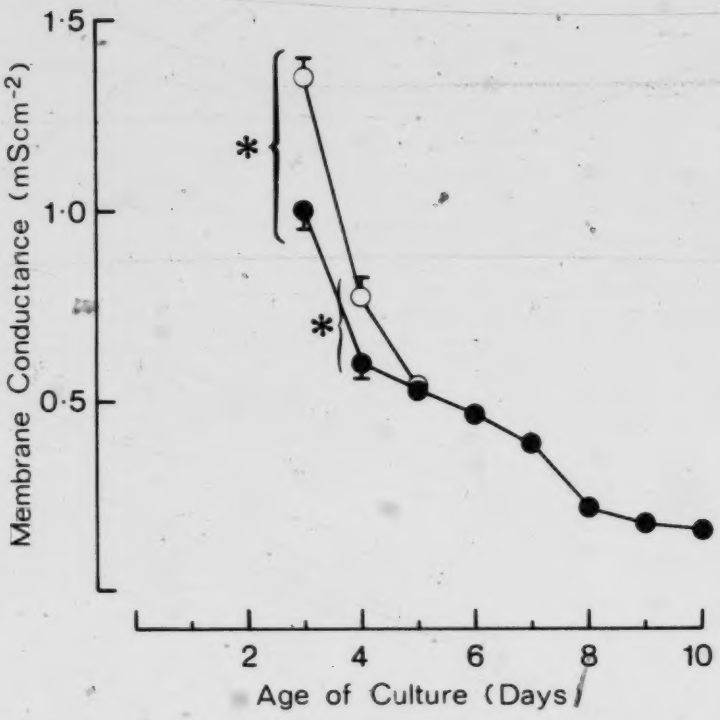


Fig. 44. Effect of verapamil on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to 5×10^{-6} verapamil HCl in BSS. Each point is the mean ± SEM of 30 measurements. Test points were not significantly different from the control at $P > 0.05$ using Student's paired *t*-test unless otherwise indicated. The fibres were the same as in Fig. 43. * $P < 0.001$.

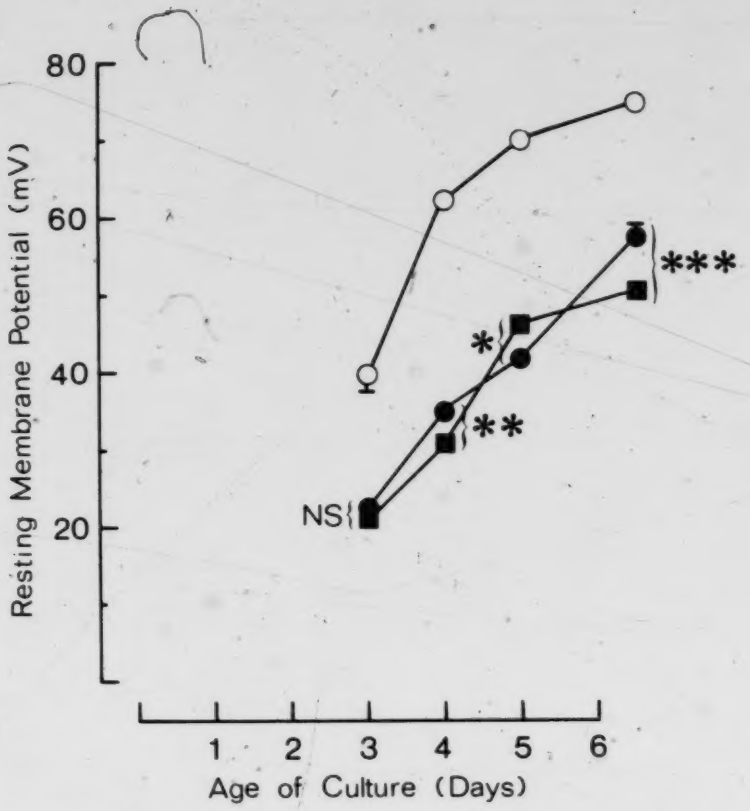
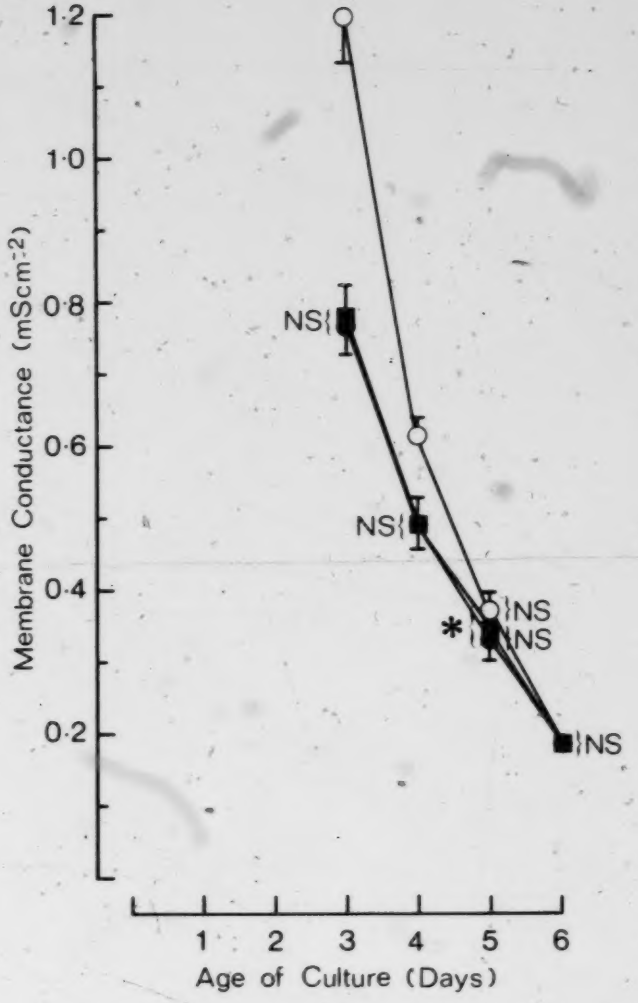


Fig. 45. Effect of removal of external sodium and then verapamil plus zero sodium solution on E_m during early development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to choline chloride plus 10^{-3}M d-TC solution (●). The same fibres were then tested again 15 minutes after changeover to choline chloride and 10^{-3}M d-TC solution plus 5 x 10^{-6}M verapamil HCl (■). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. *$P < 0.02$, **$P < 0.01$, ***$P < 0.005$.

Fig. 46. Effect of removal of external sodium and then verapamil plus zero sodium solution on G_m during early development of chick muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to choline chloride plus 10^{-3}M d-TC solution. The same fibres were then tested again 15 minutes after changeover to choline chloride and 10^{-3}M d-TC solution plus 5×10^{-6}M verapamil HCl (■). Each point is the mean ± SEM of 30 measurements. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. * $P < 0.005$. The fibres were the same as in Fig. 45.


1·2
1·0
0·8
0·6
0·4
0·2
Membrane Conductance (mScm⁻²)
NS
NS
NS
NS
*
NS
1
2
3
4
5
6
Age of Culture (Days)

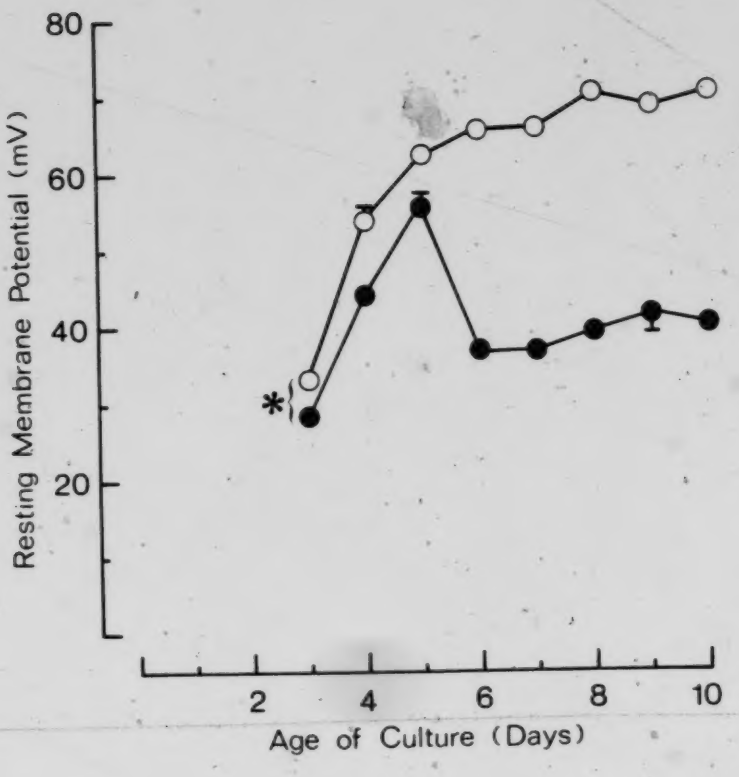


Fig. 47. Effect of veratridine on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to 10^{-5}M veratridine HCl in BSS (●). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control at $P < 0.001$ using Student's paired *t*-test unless otherwise indicated.
* $P < 0.005$.

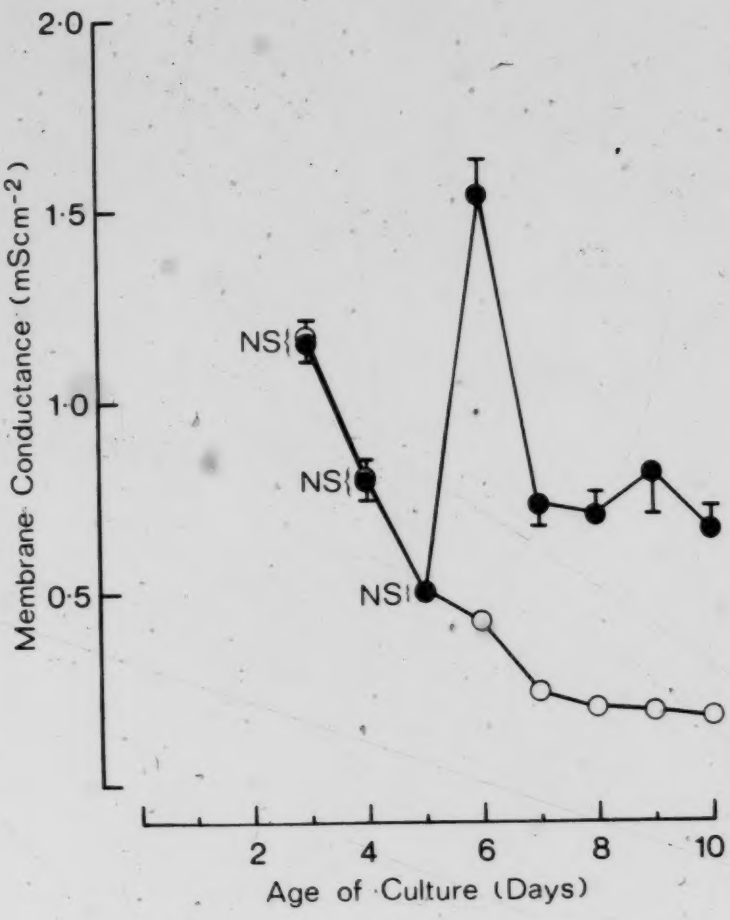


Fig. 48. Effect of veratridine on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to 10^{-5}M veratridine HCl in BSS. Each point is the mean ± SEM of 30 measurements. Each test point is significantly different from the control at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. The fibres were the same as in Fig. 47.

alone was applied to individual fibres. When applied to fibres which had been previously hyperpolarized to a resting potential of -80 mV to avoid the complication of sodium inactivation (Hodgkin and Huxley, 1952c), CTX produced depolarization of most fibres tested during the 20 second period of application (Figure 49) during which time 10 µl was discharged to surround the fibre close to the recording electrode. Membrane input resistance was monitored by the passage of constant current pulses sufficient to depolarize the fibres to -40 mV at a rate of one per second. In older fibres the current strength was adjusted to produce a depolarization just below threshold for that fibre. Depolarizing current pulses were used to activate the regenerative mechanism if it was present since CTX does not produce any effect without prior activation of the transient sodium channel (Narahashi *et al.*, 1969). Depolarization occurred and membrane resistance was reduced by CTX but both responses were more pronounced in older fibres than in younger fibres. Often the application of toxin caused the generation of an action potential - again in older fibres rather than in younger fibres. When CTX produced no response in young fibres (3 to 4 days old), no evidence of regenerative activity in such fibres was found. The application of BSS alone produced either no change in membrane potential or a slight hyperpolarization and membrane input resistance was unaffected (Figure 50).

The effect of CTX on the immature regenerative system was studied and in 3 to 5 day old cells, the application of the toxin produced an increase in the rate of rise of the regenerative response (Figure 51). The effect on action potential generation was not itself studied since the application of toxin generally induced the firing of an action

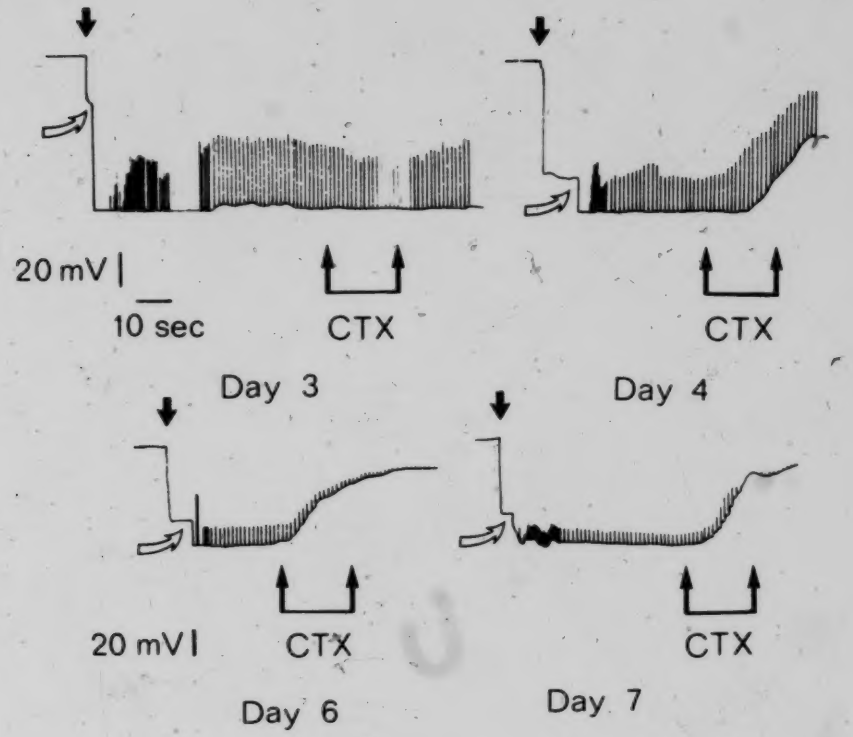


Fig. 49. Effect of condylactis toxin on E_m during development of chick muscle in culture. (↓) indicates penetration, (⇗) application of bias voltage to hyperpolarize the fibres to -80 mV. (⊔) application of 10 µl of 0.2 mg/ml CTX in BSS ejected from a microperfusion pipette for 20 seconds to surround the fibre at the recording electrode. In the immature fibre on day 3 this did not significantly affect E_m or R_{in}. On day 4 however, application of CTX produced a depolarization of the fibre after 15 seconds. In the more mature fibres on days 6 and 7, application of CTX initiated a rapid depolarization within 5 seconds accompanied by a large reduction in R_{in}. The small hump observed on day 7 is a slight contracture of the fibre.

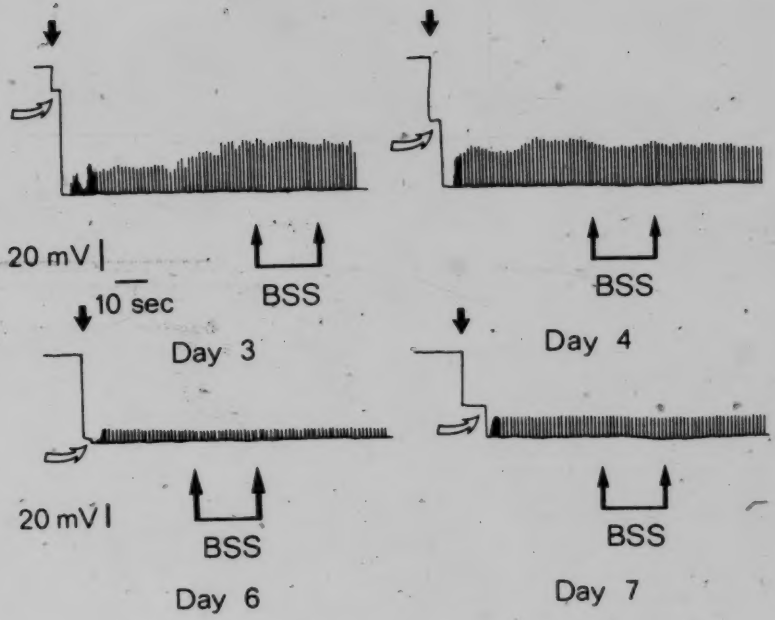
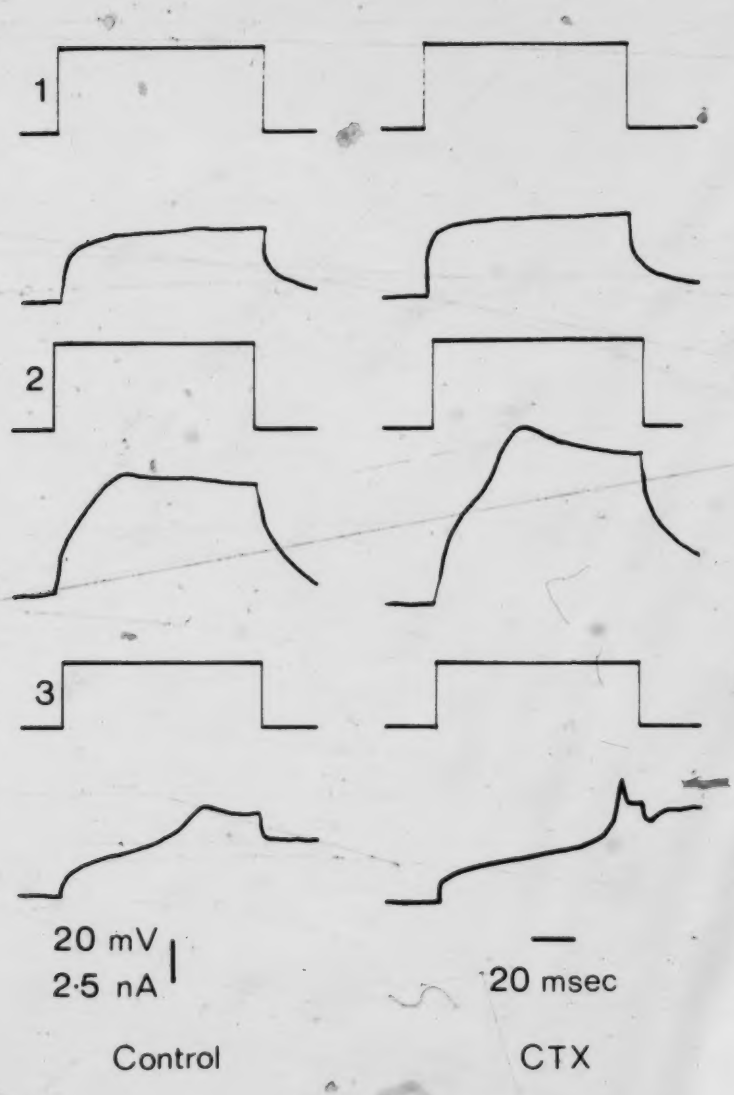


Fig. 50. Effect of BSS on E_m during development of chick muscle in culture. (↓) indicates penetration, (⇗) application of bias voltage to hyperpolarize the fibres to -80 mV, (↳) application of 10 µl of BSS ejected from a microperfusion pipette for 20 seconds to surround the fibre at the recording electrode. BSS did not significantly affect E_m or R_{in} during or immediately after application in fibres at all stages of development.

Fig. 51. Effect of condylactis toxin on regenerative activity of chick muscle in culture. Responses to the application of a 100 msec depolarizing current pulse were recorded before and after the application of 10 µl of 0.2 mg/ml CTX in BSS from a microperfusion pipette for 20 seconds to surround the fibre at the recording electrode. 1. A 3 day old fibre which produced no regenerative response to depolarization and after the application of CTX no alteration in the voltage change in response to the same current pulse occurred. 2. A 3 day old fibre which produced a small, slow regenerative response at the height of the depolarization. When the same current pulse was applied after the microperfusion of CTX the amplitude and maximum rate of rise of this regenerative activity was increased. 3. A 5 day old fibre which, during the application of a depolarizing current pulse, produced a greater regenerative response than that observed in the 3 day fibre but which was also slow. When the same current pulse was applied after the microperfusion of CTX, the amplitude and maximum rate of rise of this regenerative activity increased and a definite spike was seen.


1
2
3
20 mV
2·5 nA
20 msec
Control
CTX

potential in older cells. Application of BSS to immature fibres did not increase the rate of rise of the regenerative activity.

C. Discussion

1. Resting Membrane Conductance.

i. Replacement of External Sodium. Replacement of sodium by choline resulted in depolarization and a reduction of resting membrane conductance in immature myotubes. The action on G_m fell progressively on successive days until by the sixth day in culture no alteration of G_m was detectable. The depolarization observed throughout the period of study cannot simply be predicted by the Goldman-Hodgkin-Katz equation from which a small hyperpolarization would be expected. It also cannot be attributed to an action on ion channels since the depolarization did not decrease during maturation but if anything tended to increase. Others have observed depolarization of frog, mouse and rat muscle in sodium-free solutions (Dulhunty and Horowicz, 1975; Dulhunty, 1978) and have proposed that the depolarization resulted because potassium permeability was dependent on the valency and total concentration of cations. This postulate is not borne out by the present data because if $\bar{g}K$ were reduced, then G_m would fall throughout development. For a 20 mV depolarization, the potassium permeability is required to fall to 2/3 of its original level (assuming the other variables in the Goldman-Hodgkin-Katz equation are not substantially altered). Since $\bar{g}K$ is directly proportional to P_K this would mean a reduction in G_m of 0.033 $mScm^{-2}$ - which would be detected during the course of these experiments.

A plausible alternative mechanism involves electrogenic pumping of

sodium via Na^+-K^+ ATPase which has an absolute requirement for internal sodium to maintain activity (Glynn and Karlish, 1975). If the concentration gradient for sodium were reversed, e.g. by substituting externally with impermeant cations, then internal sodium would tend to leave the fibres. Enough may be lost so that Na^+-K^+ ATPase can no longer function adequately to maintain the resting membrane potential by the electrogenic extrusion of sodium. Sodium activity was found to fall rapidly by 4 mM within 5 minutes of reducing $[Na]_o$ to one tenth normal in a crab muscle preparation, which would support this hypothesis (Vaughan-Jones, 1976). However membrane potential was relatively unaffected, and Vaughan-Jones suggested that if the reduction in sodium activity represented sodium efflux, then the efflux must be active and coupled to preserve electrical neutrality. In the case of avian muscle in culture however it is possible that the rapid depolarization is a result of the loss of electrogenic pumping of sodium against its concentration gradient. As already emphasized elsewhere the lack of response in the L6 clone (Kidokoro, 1975a) may reflect the altered membrane in this cell line. The depolarization noted by Dryden *et al.* (1974) is encouraging but cannot be directly compared with this study since both Na^+ and Cl^- were removed from the external solution. Depolarization has now been observed when both ions were replaced separately (Chapter VI), so the combination of the two would also be expected to produce depolarization.

The magnitude of the conductance blocked by replacement of sodium by choline corresponds closely with the previously unidentifiable conductance observed in immature fibres (0.35 $mScm^{-2}$ on day 3; 0.2 and 0.05 $mScm^{-2}$ on days 4 and 5 respectively). Both conductances are no

longer detectable after the fifth day in culture. Therefore it appears that there is a substantial resting sodium conductance in immature chick myotubes which diminishes rapidly as resting membrane potential rises and is no longer demonstrable after 5 days in culture. This would account for the reduction in P_{Na}/P_K ratio observed by others in chick and rat cultures (Dryden *et al.*, 1974; Ritchie and Fambrough, 1975).

ii. Application of Verapamil. Verapamil caused a similar reduction in membrane conductance and a similar depolarization although less in magnitude to that observed in the sodium-free solution. Depolarization is not normally observed with verapamil (Reuter, 1973) but this may be a manifestation of the relative immaturity of the myotube membrane in tissue culture. The mechanism of action does not appear to involve membrane ion channels since it is unaccompanied by alterations in G_m throughout development. An action of verapamil on Na^+-K^+ ATPase has not been reported or indeed sought and an explanation for the depolarization must await further investigation.

Verapamil has normally been used to block ionic channels opened during activity in excitable membranes which apparently allow the ingress of calcium (Reuter, 1973) although the slow current channel in cardiac muscle allows the passage of both calcium and sodium (Fleckenstein *et al.*, 1969). Because there is no evidence of a resting calcium conductance in chick myotubes (Chapter VII) it is difficult to accept that verapamil is inhibiting such a conductance.

Reduction in transient sodium currents by both verapamil and D600 has been observed in squid axon (Baker *et al.*, 1973a; 1973b) and in frog

and crayfish muscle (Van der Kloot and Kita, 1975) although relatively high concentrations were employed (10^{-4} to 10^{-3}M). However micromolar concentrations of D600 reduced the maximum rate of rise of the cardiac action potential both *in vivo* and in tissue culture (Rosen *et al.*, 1974; Bayer *et al.*, 1975; Galper and Catterall, 1978). In addition Galper and Catterall (1979) found that activation of sodium channels of neuroblastoma cells by veratridine was inhibited by D600. There is therefore evidence that verapamil and its methyoxy derivative can affect the transient sodium channel in excitable membranes. Thus it is not inconceivable that verapamil may interact with resting sodium channels if these are present in the membrane. Because the action of verapamil on G_m parallels that of replacement of sodium and since it is prevented by the prior removal of sodium from the bathing solution this supports such a mechanism. Verapamil can therefore compete successfully with sodium for a resting conductance which is present in immature myotubes. As this pathway is lost or becomes inaccessible during maturation, then the action of verapamil on resting conductance disappears simultaneously.

iii. Application of Tetrodotoxin. Tetrodotoxin reduces resting conductance and causes hyperpolarization in squid axon (Freeman, 1969; Narahashi, 1972) but it had no effect on either parameter in chick myotubes in culture. However, in this system, regenerative potential sensitivity to TTX does not appear until the sixth day of culture. Before then regenerative activity is susceptible only to calcium antagonists (Chapter III).

Since the action of TTX on the squid axon was attributed to blockade of resting sodium influx through transient channels then it is not

surprising that the toxin produced no effect in immature fibres. In older cultures, when the receptor for TTX is present (Kano and Yamamoto, 1977; Spector and Prives, 1977; Chapter III), there is no longer a discernable resting sodium conductance (*vide supra*). Therefore again TTX would produce no apparent effect on E_m or G_m. However the resting membrane potential (≃70 mV) of chick myotubes in culture as in adult avian and mammalian muscle is less than E_K (≃-90 mV). Thus there must be an influx of sodium which apparently does not contribute to G_m and is not blocked by the application of TTX. This route of sodium permeation in chick myotubes cannot be through the transient sodium channel.

2. Transient Sodium Channel.

i. Application of Veratridine. Veratridine activates sodium channels in adult nerve and muscle (Shanes, 1958; Ulbricht and Flacke, 1965) and also in cultures of both types of tissue (Catterall and Nirenberg, 1973). Catterall (1975b, 1975c) has further demonstrated that the channels activated by veratridine are identical to those which are normally activated by depolarization i.e. the transient sodium channels. The failure of veratridine to increase resting membrane conductance or to cause pronounced depolarization in immature chick myotubes must therefore be because the receptor for the drug (either the m^3 gate itself or a binding site closely associated with it) is not then present in the membrane or is inaccessible. However by day 6 this moiety appears or is now functional because the application of veratridine produced a large depolarization and a significant increase in membrane conductance. It is of interest to note that day 6 is when chick myotubes first exhibit sensitivity to TTX (see following section).

ii. Application of Condylactis Toxin. In contrast to veratridine, condylactis toxin caused depolarization and a reduction of input resistance or enhanced regenerative activity in chick myotubes throughout development, although the action of the toxin on E_m and R_{in} was not evident in the youngest fibres (day 3). As myogenesis proceeded the response to application of CTX became more rapid and increased in magnitude. CTX has been proposed to act at or near the inactivation mechanism (h process) of the transient sodium channel (Narahashi *et al.*, 1969). These results would indicate that inactivation occurs and the receptor for CTX is available for reaction in chick myotubes throughout maturation in culture. The increase in response to CTX which occurs may result from the increased ion gradient (Dryden *et al.*, 1974; Ritchie and Fambrough, 1975).

From the above data and the known development of sodium channels in chick myotubes in culture two alternative hypotheses for the development of regenerative activity can be considered. One proposes a developmental elaboration of simple regenerative channels by the addition of gating molecules. The other suggests that these channels are removed *in toto* from the membrane as a consequence of turnover to be replaced by the more complicated transient sodium channel (see Chapter X for a discussion of ion channel turnover). The simple channels possess only the inactivation mechanism and are sensitive to the action of CTX but the complex transient channel possesses receptors for CTX, TTX and veratridine. It is conceivable that the resting simple channel may allow the permeation of sodium in the most immature fibres. Because the disappearance of $\bar{g}Na$ is simultaneous with the appearance of slow regenerative potent-

ials this feature of the resting channels must be lost during maturation. Further the same channel may allow the passage of calcium in addition to sodium when activated by depolarization since regenerative responses in young myotubes are apparently due to both ions (Land *et al.*, 1973). This is supported in the present work by the observation that a small increment in stimulus strength could restore slow regenerative responses which had previously been abolished by the addition of cobalt, manganese or lanthanum. At present it is impossible to resolve the two hypotheses of regenerative channel development on the basis of current electrophysiological and biochemical data (see Chapter X).

IX. EFFECTS OF ANTHRACENE-9-CARBOXYLIC ACID AND 3,4-DIAMINOPYRIDINE ON OTHER ION CONDUCTANCES IN IMMATURE FIBRES

A. Introduction

Anthracene-9-carboxylic acid and 3,4-diaminopyridine have been used to block chloride and potassium channels respectively in mature excitable membranes (Bryant and Morales-Aguilera, 1971; Palade and Barchi, 1977b; Kirsch and Narahashi, 1978). However in immature chick muscle in culture both drugs produced an increase in resting membrane conductance instead of the expected fall in G_m. Because the increase in membrane conductance was accompanied by depolarization, the ion or ions involved could only be sodium or calcium. If resting membrane conductance to potassium or chloride were increased, then hyperpolarization would result.

Because 10^{-6}M verapamil could prevent the cytotoxic effect of 9-AC in immature fibres, it was thought that the depolarization and increase in G_m were due to an increase in membrane conductance to calcium (Dryden and Thomson, 1979). Subsequently no evidence of a resting calcium conductance was found, but a resting sodium conductance was discovered which is present only in immature fibres (Chapter VIII). The finding that verapamil could block the resting sodium conductance indicated that an increase in resting membrane conductance to sodium could also account for the effects of 9-AC. Accordingly experiments were performed in which external sodium was replaced by choline, or verapamil was included in the medium and then the effects of 9-AC and 3,4-DAP were tested in fibres up to the sixth day of culture to distinguish between the two mechanisms.

B. Results

Figures 52 and 53 illustrate the effect of 9-AC after external sodium had been replaced by choline. Resting membrane potential was reduced by choline alone but there was a further reduction of about 10 mV when 9-AC was added to the sodium-free bathing medium (Solution D). The resting sodium conductance was slightly greater than found previously (Figure 40) but had disappeared by day 6. The addition of 9-AC no longer affected G_m in the early stages of development until the sixth day of culture. At this stage, 9-AC produced a reduction in G_m of 0.16 $mScm^{-2}$ after removal of external sodium which was similar to that found when the action of 9-AC was investigated in normal BSS (Figure 26). Similar results were obtained when verapamil was employed to block resting sodium conductance prior to the addition of 9-AC (Figures 54 and 55).

When 3,4-DAP was used in combination with solution D, membrane potential was not greatly affected on days 3 and 4 but on days 5 and 6 a 10 mV depolarization further to that caused by choline was found (Figure 56). Membrane conductance was not affected by the addition of 3,4-DAP to choline-treated fibres until the sixth day of culture (Figure 57) when a reduction in G_m of 0.07 $mScm^{-2}$ was produced.

Finally the effect of verapamil on the action of 3,4-DAP was investigated. Resting membrane potential was depressed by 3,4-DAP alone and then further depressed by the addition of 5×10^{-6}M verapamil (Figure 58). The 3,4-DAP-activated conductance observed during early development was abolished by the subsequent addition of verapamil and G_m was even reduced to below control levels (Figure 59). On the sixth day of culture, 3,4-DAP reduced G_m but the addition of verapamil caused no further alteration in resting membrane conductance.

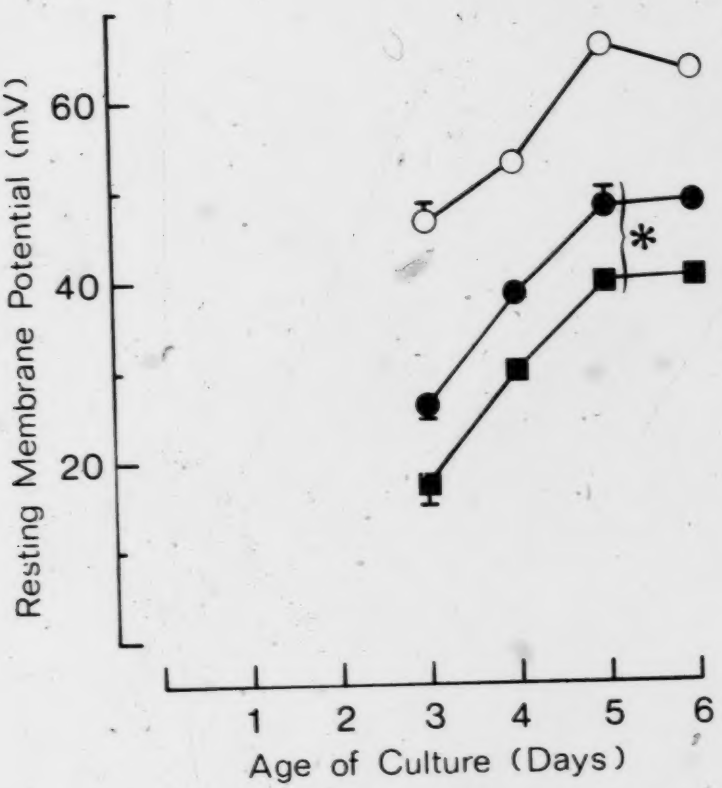
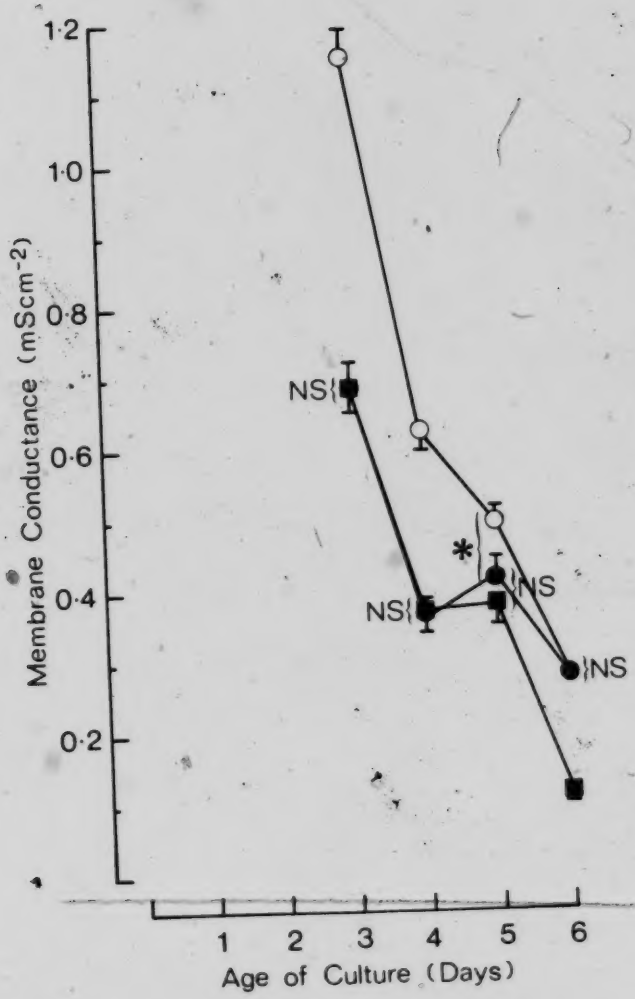


Fig. 52. Effect of choline chloride plus 10^{-3}M d-TC and then choline chloride and 10^{-3}M d-TC plus 9-AC on E_m during early development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to choline chloride plus 10^{-3}M d-TC solution (●). The same fibres were then tested again 15 minutes after changeover to choline chloride and 10^{-3}M d-TC solution plus 5×10^{-5}M 9-AC (■). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired *t*-test unless otherwise indicated. * $P < 0.005$.

Fig. 53. Effect of choline chloride plus 10^{-3}M d-TC and then choline chloride and 10^{-3}M d-TC plus 9-AC on G_m during early development of chick muscle in culture. Control values (○) were measured in BSS and the test values were measured in the same cells 15 minutes after changeover to choline chloride plus 10^{-3}M d-TC solution (●). The same fibres were then tested again 15 minutes after changeover to choline chloride and 10^{-3}M d-TC solution plus 5×10^{-5}M 9-AC (■). Each point is the mean ± SEM of 30 measurements. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ using Student's paired *t*-test unless otherwise indicated. * $P < 0.005$. The fibres were the same as in Fig. 52.


1·2
1·0
0·8
0·6
0·4
0·2
Membrane Conductance (mScm⁻²)
NS
NS
*
NS
NS
1
2
3
4
5
6
Age of Culture (Days)

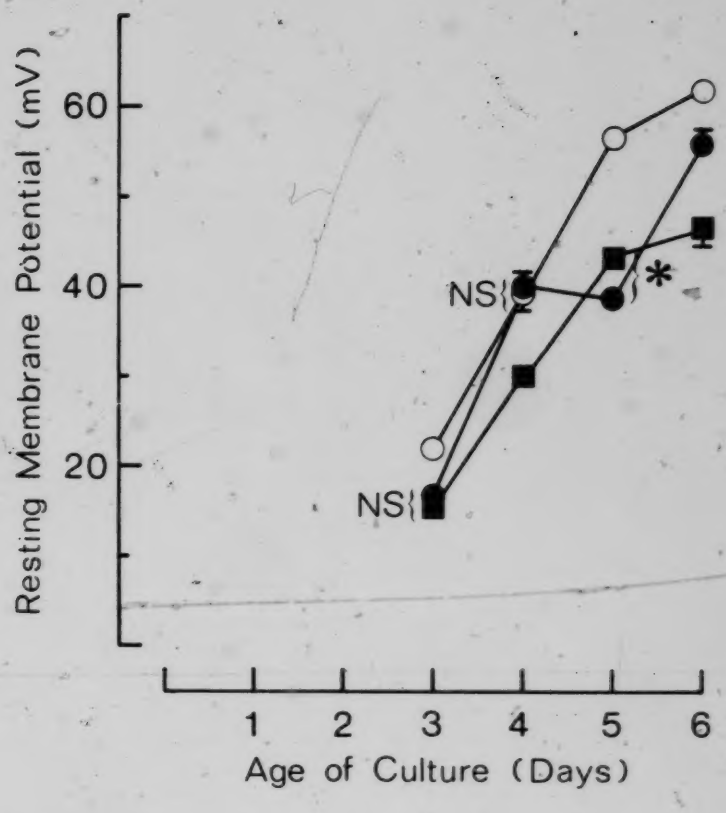


Fig. 54. Effect of verapamil and then verapamil plus 9-AC on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to 5×10^{-6}M verapamil HCl in BSS (●). The same fibres were then tested again 15 minutes after changeover to 5×10^{-6}M verapamil HCl plus 5×10^{-5}M 9-AC in BSS (■). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. The fibres were the same as in Fig. 43.
* $P < 0.02$.

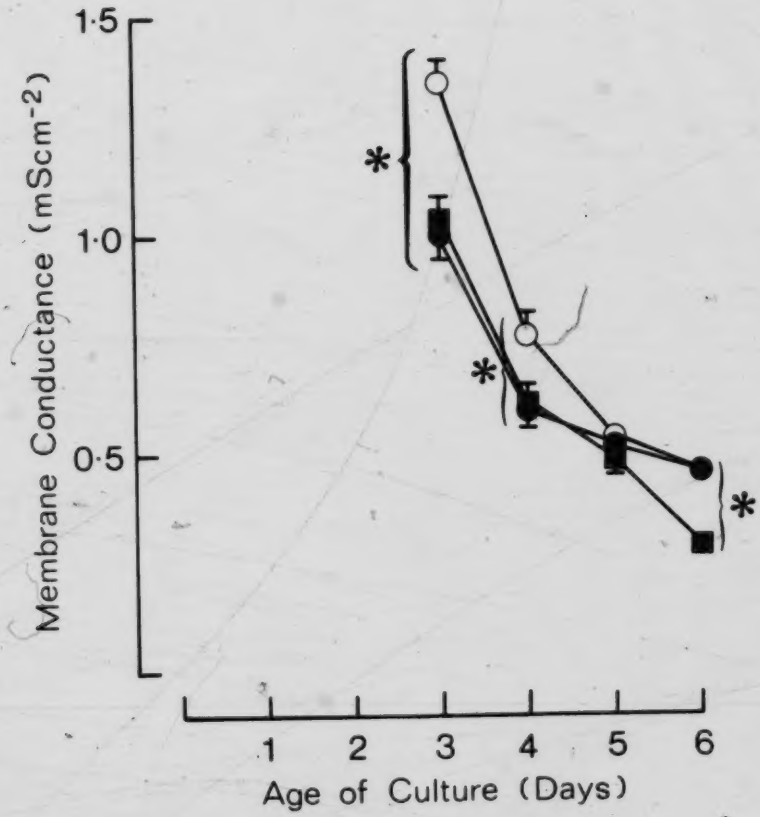


Fig. 55. Effect of verapamil and then verapamil plus 9-AC on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values were measured in the same cells 15 minutes after changeover to 5×10^{-6}M verapamil HCl in BSS (●). The same fibres were then tested again 15 minutes after changeover to 5×10^{-6}M verapamil HCl plus 5×10^{-5}M 9-AC in BSS (■). Each point is the mean ± SEM of 30 measurements. Test points were not significantly different from the control or each other on the same day at $P > 0.05$ (Student's paired *t*-test) unless otherwise indicated. The fibres were the same as in Fig. 43. * $P < 0.001$.

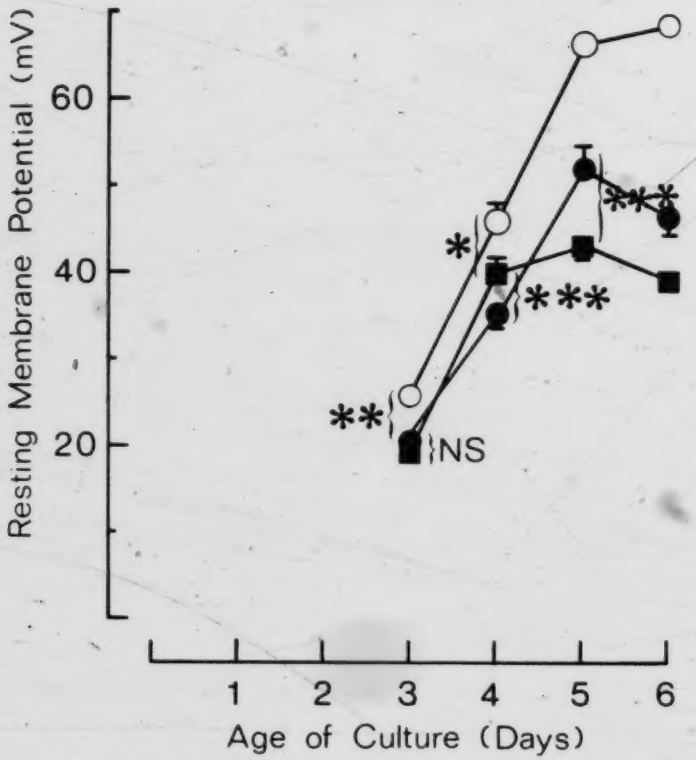


Fig. 56. Effect of choline chloride plus 10^{-3}M d-TC and then choline chloride and 10^{-3}M d-TC plus 3,4-DAP on E_m during early development of chick muscle in culture. Control values (○) were measured in BSS and the membrane potentials of the same cells were then measured 15 minutes after changeover to choline chloride plus 10^{-3}M d-TC solution (●). The same fibres were then tested again 15 minutes after changeover to choline chloride and 10^{-3}M d-TC solution plus 10^{-4}M 3,4-DAP (■). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ using Student's paired *t*-test unless otherwise indicated. * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$.

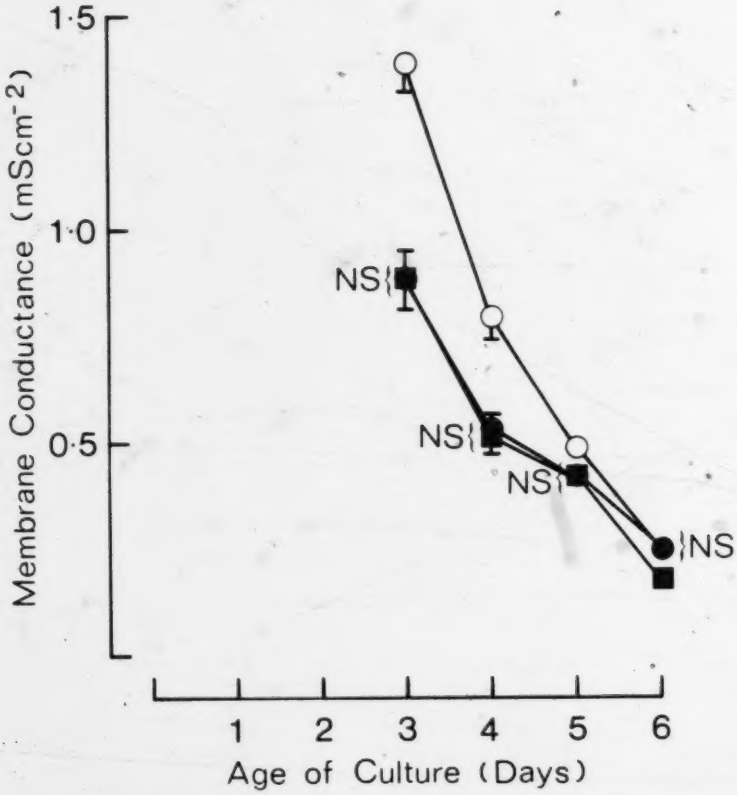


Fig. 57. Effect of choline chloride plus 10^{-3}M d-TC and then choline chloride and 10^{-3}M d-TC plus 3,4-DAP on G_m during early development of chick muscle in culture. Control values (○) were measured in BSS and the test values (●) were measured in the same cells 15 minutes after changeover to choline chloride plus 10^{-3}M d-TC solution. The same fibres were then tested again 15 minutes after changeover to choline chloride and 10^{-3}M d-TC solution plus 10^{-4}M 3,4-DAP (■). Each point is the mean ± SEM of 30 measurements. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired t-test) unless otherwise indicated. The fibres were the same as in Fig. 56.

Fig. 58. Effect of 3,4-DAP and then 3,4-DAP plus verapamil on E_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values were measured in the same cells 15 minutes after changeover to 10^{-4}M 3,4-DAP in BSS (●). The same fibres were then tested again 15 minutes after changeover to 10^{-4}M 3,4-DAP plus 5 x 10^{-6}M verapamil HCl in BSS (■). Each point is the mean ± SEM of 30 observations. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated.
* $P < 0.005$.

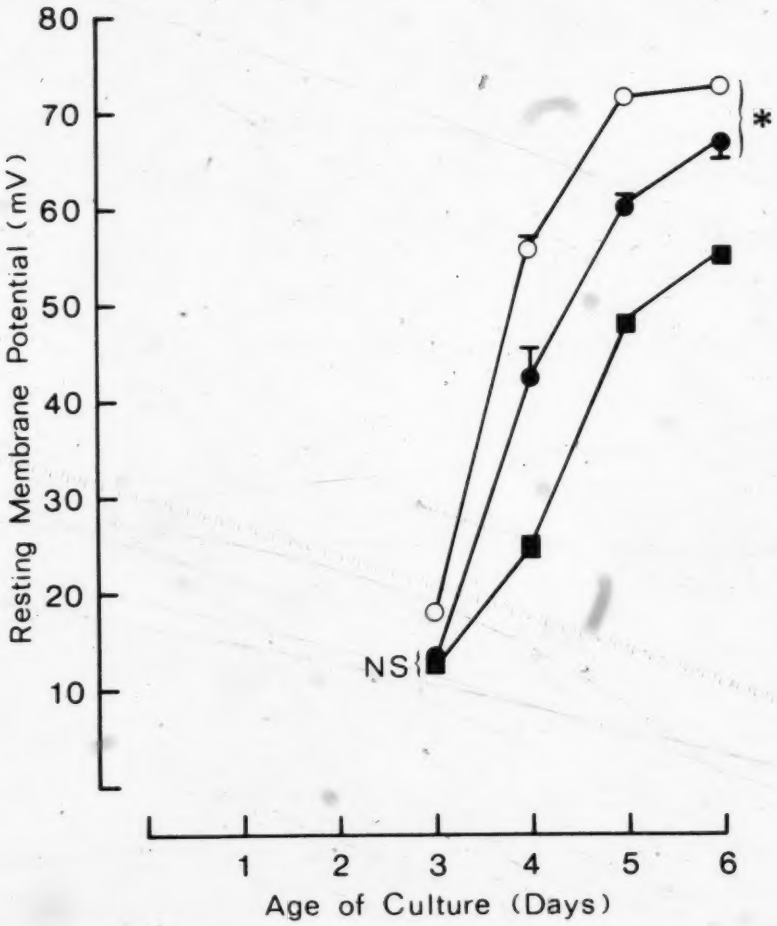
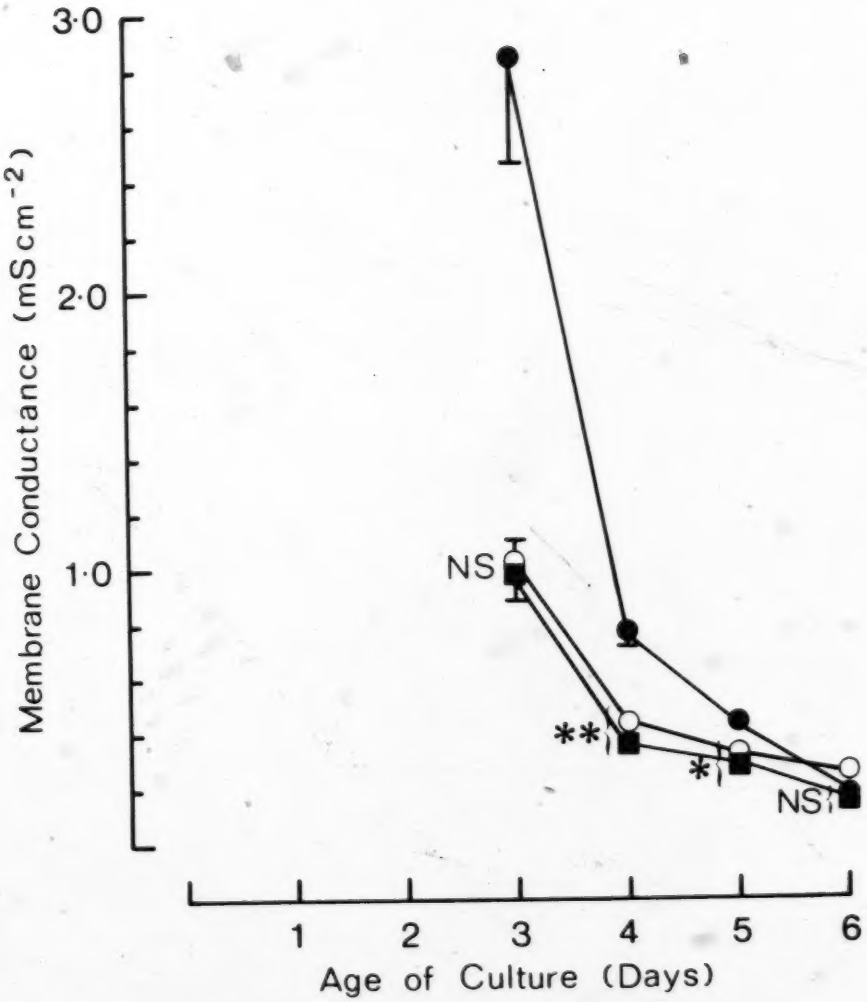

80
70
60
50
40
30
20
10
Resting Membrane Potential (mV)
*
NS
1
2
3
4
5
6
Age of Culture (Days)

Fig. 59. Effect of 3,4-DAP and then 3,4-DAP plus verapamil on G_m during development of chick muscle in culture. Control values (○) were measured in BSS and the test values were measured in the same cells 15 minutes after changeover to 10^{-4}M 3,4-DAP in BSS (●). The same fibres were then tested again 15 minutes after changeover to 10^{-4}M 3,4-DAP plus 5 x 10^{-6}M verapamil HCl in BSS (■). Each point is the mean ± SEM of 30 measurements. Each test point is significantly different from the control and from each other on the same day at $P < 0.001$ (Student's paired *t*-test) unless otherwise indicated. The fibres were the same as in Fig. 58. * $P < 0.05$, ** $P < 0.005$.


3·0
2·0
1·0
Membrane Conductance (mS cm⁻²)
NS
**
*
NS
1
2
3
4
5
6
Age of Culture (Days)

C. Discussion

When immature chick myotubes (3 to 5 days old) are exposed to BSS containing either 9-AC or 3,4-DAP then depolarization and a pronounced increase in resting membrane conductance is found. This cannot be associated with the respective blockade of chloride or potassium channels which occurs in adult muscle (Bryant and Morales-Aguilera, 1971; Kirsch and Narahashi, 1978) and in the more mature chick myotubes (Chapters V and VI). However when the two drugs were used in combination with a sodium free solution or in BSS containing verapamil, then a reduced depolarization was found accompanied by no alteration in G_m. The reduction in resting membrane conductance observed on day 6 with both drugs in either solution can be attributed to blockade of $\bar{g}Cl$ and $\bar{g}K$ as noted before in BSS.

The observation that verapamil in micromolar concentrations could prevent or reverse the increase in G_m caused by 9-AC or 3,4-DAP would superficially favour an increase in $\bar{g}Ca$ because of the known blocking action of verapamil on calcium currents in adult excitable membranes (Reuter, 1973). But as discussed earlier (Chapter VIII) verapamil has been shown to reduce sodium currents in excitable tissue and in immature chick myotubes verapamil inhibited a resting sodium conductance. This finding together with the observation that substitution of sodium in the bathing fluid mimics the results obtained with verapamil would favour the alternative hypothesis of a drug induced increase in the existing membrane conductance to sodium. The progressive reduction in the action of 9-AC and 3,4-DAP on G_m would therefore be predictable on the basis of the known fall in $\bar{g}Na$ of immature myotubes.

The possibility that calcium may penetrate the immature myotube membrane through the sodium conductance channel must be considered. Because there was no detectable increase in G_m of fibres exposed to 9-AC and 3,4-DAP after replacement of sodium, this indicates that very little if any calcium can penetrate through the channels in the absence of sodium. There was additionally no evidence of calcium toxicity at the time of electrophysiological measurement. However at longer time intervals, 9-AC (and zinc) produce membrane damage which is postulated to be due to excess internal calcium (Chapter VI). Permeation of calcium by this route could be involved in the production of cytotoxicity, but does not appear to be involved in the earlier electrophysiological response to 9-AC (and zinc). The cytotoxic effect of 9-AC (and zinc) would therefore be expected to disappear in parallel with the loss of the resting sodium conductance. An increase in the ability of the SR to accumulate calcium would produce the same result if the calcium was entering by some other means. If calcium was entering via the sodium conductance channel, then 3,4-DAP should also cause evidence of cytotoxicity in immature myotubes. However the action of 3,4-DAP on myotube membrane integrity was not investigated.

The depolarization produced by 9-AC and 3,4-DAP in the absence of external sodium which is unaccompanied by a concomitant alteration in G_m cannot involve an action on membrane ion channels. Because fibres were penetrated several times during the course of an experiment membrane damage could be involved in the response. However, this was discounted for the reasons discussed previously (Chapter V). The mechanism of the depolarization must therefore involve other cellular processes eg. Na^+-K^+

ATPase. It may not necessarily be due to specific drug effects but could simply reflect a gradual metabolic run down of the fibres during the course of an experiment since the duration of these experiments was 1½ to 2 hours for each culture dish examined.

The actions of anthracene-9-carboxylic acid on chick myotubes in culture are therefore more complex than is apparent in adult muscle fibres. Depending on the ion composition of the bathing fluid and the age of the myotubes, different responses to 9-AC have been observed. The predominant effect on immature myotubes is one of depolarization accompanied by an increase in resting membrane conductance; now attributed to an increase in $\bar{g}Na$. This was found when 9-AC was used in normal BSS (Figures 22 and 26), in combination with a chloride-free medium (Figures 27 and 28) or in combination with the potassium channel blocking agent, TEA (Figures 29 and 30). As the resting sodium conductance disappears so too does this action of 9-AC, to be replaced by an action on the chloride channel in the more mature myotubes. Substitution of chloride in the bathing fluid surrounding the older fibres together with the application of 9-AC uncovers an increase in $\bar{g}K$ postulated to occur through the chloride channel (Chapter VI).

The actions of 3,4-diaminopyridine on immature chick myotubes are similar to those of 4-AP. 3,4-DAP was investigated further because it is the most potent of the aminopyridines (Kirsch and Narahashi, 1978). By analogy, 4-AP is also suggested to cause depolarization and an increase in membrane conductance (Figures 16 and 17) by an increase in resting sodium conductance. It is therefore obvious that agents reputed to block specific ion channels in excitable membranes must be used with

extreme caution to investigate the development of chick muscle in culture. Preferably the primary investigation should be made by ion replacement experiments and the blocking agents used solely for confirmation of these results.

X. GENERAL DISCUSSION

A. Comparison with Non-Excitable Cells

The immature myotube formed by fusion of myoblasts undergoes development in culture to form a relatively mature muscle fibre. This transformation occurs over a short period (day 3 to day 5-6) during which the characteristics of the differentiated state appear. The levels of muscle-specific and other membrane proteins such as the Ach receptor, acetylcholinesterase, adenylate cyclase and Na^+-K^+ ATPase increase rapidly to a maximum by day 6 (Fambrough and Rash, 1971; Fluck and Strohman, 1973; Prives and Paterson, 1974; Prives, 1976; Spector and Prives, 1977). A similar accumulation of cytoplasmic proteins (i.e. myosin, creatine phosphokinase, glycogen phosphorylase, myokinase) is found which may (Shainberg and Brik, 1978) or may not (Paterson and Prives, 1973) be coupled to the membrane differentiation (Shainberg *et al.*, 1971; Hauschka, 1972; Stockdale and O'Neill, 1972). At the ultrastructural level, the myofilaments become aligned in parallel arrays (Fischman, 1970) and the sarcoplasmic reticulum develops (Ezerman, 1968).

Concomitant with the morphological and biochemical maturation, the electrophysiological properties of the membrane develop. The resting membrane potential rises rapidly to adult levels (Fischbach *et al.*, 1971; Fambrough and Rash, 1971; Powell and Fambrough, 1973; Dryden *et al.*, 1974; Ritchie and Fambrough, 1975; Spector and Prives, 1977). This is accompanied by the appearance of slow regenerative activity attributed to the influx of both calcium and sodium ions which is subsequently replaced by fast action potentials mediated by the transient sodium channel (see Chapter I, Section B.2 Transient Channels and Chapter

VIII). The present data confirms these findings but in addition reports that the resting conductances to the individual determinants of membrane potential do not vary in a similar manner. Although sodium and chloride conductances fall rapidly between days 3 and 5, the potassium conductance remains relatively constant throughout development (Figure 60).

Data on the resting membrane conductances of the unfused myoblast are missing, but the situation in 3 day myotubes cannot be dramatically different. The membrane potential of myoblasts and newly-formed myotubes is about -10 mV (Dryden *et al.*, 1974) and similarly the morphological and biochemical parameters do not change immediately after fusion (*vide supra*). On this basis, by extrapolation, the myoblast should have a high resting membrane conductance (on the order of 1 $mScm^{-2}$). The $\bar{g}Na/\bar{g}K$ ratio should be close to or in excess of 1 as indeed was found for the P_{Na}/P_K ratio (Dryden *et al.*, 1974). There should be a high chloride conductance in myoblasts and no discernable resting calcium conductance. Myoblasts are not differentiated cells although they may be committed to differentiate and have undergone their last mitosis prior to fusion (see Stockdale and Holtzer, 1961; Bischoff and Holtzer, 1969). They may therefore be compared with non-excitable cells.

Low membrane potentials of around -10 mV to -20 mV have been reported for Ehrlich ascites tumour cell (Aull, 1967; Hoffman *et al.*, 1979) red blood cells (Lassen and Sten-Knudsen, 1968; Jay and Burton, 1969) HeLa cells (Borle and Loveday, 1968), L cells (Lamb and MacKinnon, 1971; Nelson *et al.*, 1972). These are comparable to estimates of E_m in myoblasts and early myotubes (*vide supra*). Higher membrane potentials have been reported for endocrine tissue (Williams, 1970). Williams

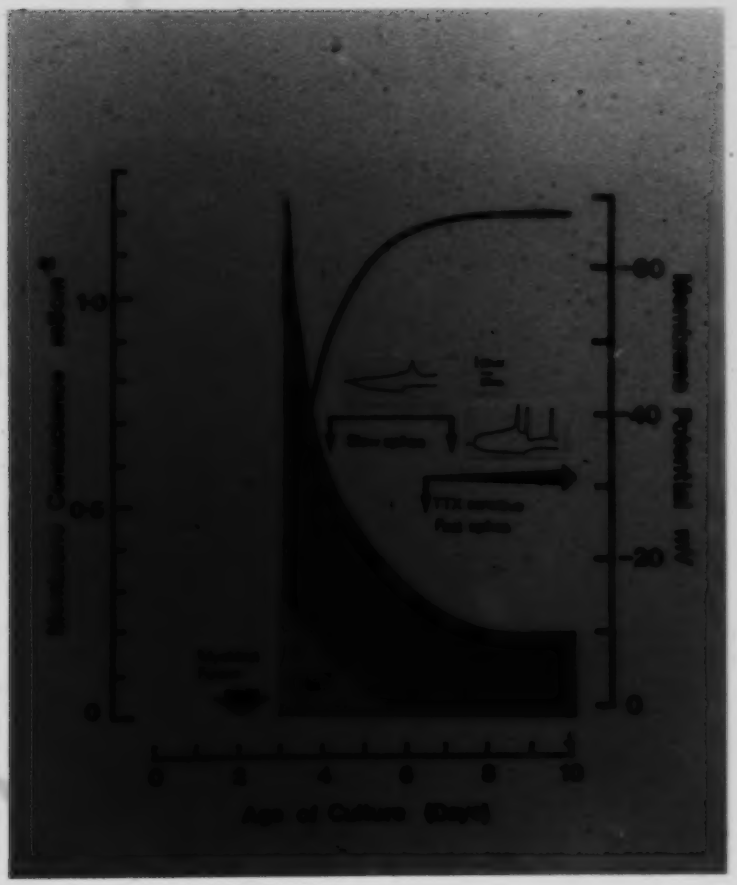


Fig. 60. Summary of changing conductances which accompany the rise of resting membrane potential during myogenesis of chick skeletal muscle in culture.

pointed out that glandular tissue contains several functionally different types of cells whose membrane properties may also be different. This may contribute to the spread of membrane potentials reported (-20 to -90 mV). Membrane conductances have only been estimated in a few cases due to the technical difficulties associated with penetration of small cells. This is illustrated by comparison of two estimates of G_m for Ehrlich ascites tumour cells. One report estimates G_m to be 42 μScm^{-2} using flux data (Hoffmann *et al.*, 1979) and the other found G_m was 14 $mScm^{-2}$ by estimation of the increase in resistance seen by the microelectrode tip after penetration of the cell (Lassen *et al.*, 1971). Lassen *et al.* (1971) concluded that the resistance measurement was dominated by leakage.

Nelson *et al.* (1972) obtained a value of 0.2 $mScm^{-2}$ for L cells assuming that the fibroblasts had a smooth surface. However, the surface of L cells is covered with densely packed filopodia and Nelson *et al.* estimated this would reduce G_m to between 0.1 $mScm^{-2}$ and 50 μScm^{-2} assuming the surface area was increased by a factor of 2 or 4. Liver, thyroid, glial cells and rabbit ileum all have exceptionally high conductances: 27, 33, 100, and 19.7 $mScm^{-2}$ (Schanne and Coraboeuf, 1966; Woodbury and Woodbury, 1963; Hild and Tasaki, 1962; Munck and Schultz, 1974). The high permeability of the thyroid was attributed to a high anion permeability in view of its active iodide transport function (Woodbury and Woodbury, 1963), and that of the ileum to the extracellular pathway for ion penetration. Munck and Schultz (1974) found that this contributed at least 80% of the total G_m which still leaves a conductance of 4 $mScm^{-2}$ which presumably is due to penetration across the

cells of the ileum. The conductances of amphibian kidney tubules (excluding the shunt pathway) have been reported to be 0.126 and 0.14 $mScm^{-2}$ in Necturus (Windhager *et al.*, 1967; Anagnostopoulos and Velu, 1974) 0.33 $mScm^{-2}$ in Chironomus (Loewenstein *et al.*, 1965) and 1.19 $mScm^{-2}$ in the newt (Hoshi and Sakai, 1967).

Although there is considerable variation, most non-excitable cells possess a relatively high resting membrane conductance similar to G_m in immature myotubes - the exceptions being the Ehrlich ascites tumour cells and L cells. L cells are a clonal cell line - and cell lines are known to exhibit abnormal electrophysiological properties (see Chapter I). The unusually low conductance of the ascites cells may similarly be a manifestation of the altered membrane properties of tumour cells.

Williams (1970) has compared the P_{Na}/P_K ratio for a variety of cell types and found that this was higher for non-excitable cells (0.03 - 0.36) compared to excitable cells (0.01). Schanne and Coraboeuf (1966) and Woodbury and Woodbury (1963) also found that P_{Na} was relatively high (0.3 and 0.12) compared to P_K. The chloride permeability of both cell types was high (P_{Cl}/P_K = 1.5 for liver). Beigelman and Shu (1972) found that $P_K : P_{Na} : P_{Cl}$ of 1.0 : 0.2 : 1.5 could predict the measured E_m based on external and internal ion concentrations. Similar data i.e. a high P_{Na}/P_K ratio and a high resting chloride permeability has been reported for L cells (Lamb and MacKinnon, 1971; HeLa cells (Borle and Loveday, 1968) Ehrlich ascites cells (Aull, 1967; Hoffmann *et al.*, 1979) and baby hamster kidney fibroblast cell line (Sachs and McDonald, 1972). High values of P_{Na}/P_K have also been observed in intestinal muscle although chloride permeability is low (Wright, 1966; Munck and Schultz,

1974). Therefore the myoblast and immature myotube are similar to non-excitable cells in the possession of a high P_{Na}/P_K ratio and a high chloride conductance ($P_K : P_{Na} : P_{Cl}$ for 3 day myotubes 1 : 1.5 : 3.5).

B. Relationship Between E_m and G_m

Figure 60 illustrates the changing conductances accompanying the rise of resting membrane potential during myogenesis of chick skeletal muscle in culture. Resting membrane conductance is initially high - apparently because of the high conductance of the early myotube membrane to sodium and chloride, As G_m falls, both $\bar{g}Na$ and $\bar{g}Cl$ diminish rapidly whereas $\bar{g}K$ remains relatively constant throughout development. The possession of a large resting chloride conductance is a feature of non-excitable cells and the reduction in $\bar{g}Cl$ may be a feature of the process of differentiation.

The question remains: do the data explain the rise in resting membrane potential? This can be investigated by fitting the individual ion conductances into the 'equivalent circuit' equation (Hubbard *et al.*, 1969):

$$V = \frac{\bar{g}KV_K + \bar{g}NaV_{Na} + \bar{g}ClV_{Cl}}{\bar{g}K + \bar{g}Na + \bar{g}Cl}$$

V = membrane potential
V_K = equilibrium potential for potassium
V_{Na} = equilibrium potential for sodium
V_{Cl} = equilibrium potential for chloride

Estimates of the internal ion concentrations of chick myotubes in culture are only available for 2 day myoblasts and 5 day myotubes (Dryden *et al.*, 1974). The corresponding values for $[Cl]_i$ were 80 mM and 39.1 mM respect-

ively (Dryden - unpublished observations) and the resting membrane potentials were -9.3 mV and -30.3 mV for myoblasts and myotubes. Substituting the conductance values for 3 day myotubes ($\bar{g}K = 0.2$; $\bar{g}Na = 0.3$; $\bar{g}Cl = 0.7$ $mScm^{-2}$) into the equation and using the internal ion concentrations for myoblasts yields a calculated membrane potential of -14.75 mV. This value is midway between the membrane potential for myoblasts and 3 day myotubes (-20.88 mV) and suggests that alterations in the membrane conductance to the major ions have already begun by day 3 in culture.

Using the conductance values for 5 day myotubes ($\bar{g}K = 0.1$; $\bar{g}Na = 0.06$; $\bar{g}Cl = 0.34$ $mScm^{-2}$) the resting membrane potential of the myotubes was calculated to be -32.3 mV which agrees well with the value determined directly. In this study the resting membrane potential of 5 day myotubes was higher (-61.4 mV) than observed by Dryden *et al.* (1974). The discrepancy may lie in the fact that the ion concentration measurements were obtained in cultures of leg muscle and leg muscle does not develop as rapidly as breast muscle (Chapter IV). If the individual ion conductances at -30 mV are obtained by interpolation from Figure 60, then the calculated membrane potential (-18.96 mV) falls far short of that measured. It would therefore appear that the internal ion concentrations of breast and leg muscle alter at different rates during myogenesis and the rates at which they reach the mature state must also differ. Thus to test the conductance values, estimates of both the internal ion concentrations and the resting membrane potentials are required for pectoral muscle.

Nevertheless, the data do not support the original hypothesis that the acquisition of a high membrane potential was due to an increase

in potassium conductance (Dryden *et al.*, 1974). The present observations support the alternative theory that as a consequence of the loss of a substantial resting sodium conductance, the potassium conductance is left as the dominant one in the determination of the resting membrane potential of chick muscle in culture.

C. Turnover of Membrane Proteins

During differentiation, the synthesis and incorporation of muscle-specific membrane proteins increases. The kinetics of accumulation, degradation and protein turnover have been worked out in detail for the acetylcholine receptor by Fambrough and colleagues (Hartzell and Fambrough, 1973; Devreotes and Fambrough, 1975; Fambrough and Devreotes, 1976, 1978; Devreotes *et al.*, 1977; Linden and Fambrough, 1979). On the basis of these experiments a model for the turnover of the acetylcholine receptor protein was proposed (Devreotes and Fambrough, 1975; Fambrough and Devreotes, 1976). Ach receptor biosynthesis was considered to be analogous to that previously suggested for the synthesis of secretory proteins (Palade, 1959, 1975). Such proteins are synthesized on polysomes bound to the endoplasmic reticulum. Further processing may then take place there and/or in the condensing vacuoles of the Golgi apparatus e.g. glycosylation prior to release from the cells by exocytosis. The only difference between integral and secretory membrane proteins is that the former are tightly associated with the lipid bilayer. If secretory-like vesicles containing these proteins were to fuse with the plasma membrane, then the proteins would be exposed to the extracellular environment (Palade, 1959; Rothman and Leonard, 1976). This process is

thought to be an assembly-line linear mechanism (Linden and Fambrough, 1979).

This hypothesis is supported by several pieces of evidence. The internal binding sites for the Ach receptor label, α-BuTX, were all membrane-associated; newly-synthesized receptors were located in the Golgi apparatus; the intracellular transport of Ach receptors and secretory proteins from their sites of biosynthesis to the surface membrane were similar i.e. transport is independent of protein synthesis but requires ATP, depends on temperature and the time required for transport is several hours (≃ 3 hours for the Ach receptor). No specific α-BuTX binding sites were found in the supernatant of centrifuged cell homogenates indicating that there is no significant population of free subunits or soluble receptor precursors. The total number of receptors which were incorporated into the surface were accounted for quantitatively by the disappearance of the precursor pool, and the absence of a lag phase in the kinetics of disappearance of the precursor also suggested that there was no large store of material feeding into the precursor pool. Similarly the kinetics of reversal of the block of incorporation (by inhibition of receptor synthesis) also indicated that there was no significant time delay between protein synthesis and association of the newly-made receptors with the precursor population.

Once present in the extracellular membrane, the acetylcholine receptors remain there for a period before being degraded. Degradation is a first order i.e. random process with a $t_{\frac{1}{2}}$ of about 22-24 hours, similar to that for extrajunctional receptors in adult muscle (Berg and Hall, 1974; Chang and Huang, 1975). Degradation is thought to involve intern-

alization of the receptors by phagocytosis since it did not occur in homogenates or isolated membrane preparations. This is followed by the formation of phagolysosomes and the subsequent breakdown of the protein in secondary lysosomes. The turnover of the receptor has been suggested to reflect the turnover and cellular degradation of muscle membrane proteins and therefore that of the membrane itself. If so, this means that about 5% of the membrane with the accompanying protein is turned over daily (Devreotes *et al.*, 1977).

In the absence of evidence to the contrary one can suppose that the above mechanism pertains to other integral membrane proteins including the turnover of the transient sodium channel. This component of the mature muscle membrane is not present in its mature state in immature myotubes but appears by day 6 in culture. The mature form of the transient sodium channel may not be manufactured during the initial stages of differentiation (unlike other membrane proteins such as the acetylcholine receptor or acetylcholinesterase) and therefore only appears in the membrane at a later time.

Possibly as a consequence of turnover, the transient sodium channel replaces the immature form - the slow regenerative channel which is removed *in toto* from the membrane and degraded. Alternatively the appearance of the transient sodium channel may mark the elaboration of the pre-existing slow regenerative channel by the addition of gating molecules and the TTX sensitive-moiety (see Chapter VII).

The implicit assumption that these ion channels are at least partially protein is supported by several pieces of evidence. Messenger RNA can induce the appearance of transient sodium channels in cultures of

cardiac myoblasts (McLean *et al.*, 1976), and the inhibition of protein synthesis by cycloheximide prevents the appearance of the transient sodium channel in heart cell cultures (Nathan and DeHaan, 1978). In denervated muscle, the appearance of TTX-resistant spikes is prevented by the inhibition of protein synthesis suggesting that a new population of channels appears after denervation (Grampp *et al.*, 1972). Lastly, the proteolytic enzyme mixture, pronase, when perfused internally in squid axon, degrades the moiety responsible for the inactivation (h) process (Armstrong *et al.*, 1973).

D. Trophic Effects on the Developing Muscle Membrane

During myogenesis in culture, differentiation is incomplete since the myotubes do not exhibit the fully differentiated characteristics of adult innervated muscle. One example of this is the distribution of acetylcholine receptors. In cultured muscle the receptors are spread over the entire membrane (Vogel *et al.*, 1972) similar to the distribution of extrajunctional receptors in denervated muscle (Axelsson and Thesleff, 1959; Miledi, 1960). Although there are clusters of receptors or "hot spots" formed both in culture (Frank, 1979) and in denervated muscle (Albuquerque and McIsaac, 1970; Ko *et al.*, 1977) the receptors are not confined to one region of the membrane. The biochemical and electrophysiological properties of acetylcholine receptors in culture are very similar to those of extrajunctional receptors but both differ from those of junctional receptors (see Edwards, 1979). There has been controversy over the role that loss of muscle activity plays in the changes due to denervation but most workers are agreed that a neurotro-

phic factor is also important in regulating the distribution of acetylcholine receptors (Lavoie *et al.*, 1977; Guth and Albuquerque, 1978).

The electrophysiological properties of the acetylcholine receptor have recently been demonstrated to change during innervation *in vivo* (Sakmann and Brenner, 1978). Embryonic receptors were similar to extrajunctional receptors since the mean channel open time was long (4 msec). However after innervation had occurred, the mean channel open time was reduced to 1 msec - identical to that for junctional receptors in adult innervated muscle. It was suggested that there were two separate populations of receptor, one with a long channel open time and one with a short channel open time. The kinetics indicated that the proportions of the two populations changed during synaptogenesis.

Neurotrophic effects on the appearance of the transient sodium channel in cultured muscle have also been demonstrated recently. These have been compared with the alterations in the regenerative process which occur on denervation (see Chapter I, Section B.2 Transient Channels). The passive membrane properties of muscle are known to alter after denervation and changes in the opposite direction have been observed after innervation *in vitro* (see Chapter VI). Therefore it is not surprising that the component conductances of 10 day myotubes closely resemble that of denervated rather than innervated muscle (see Chapter VI). It is thus predicted that co-culturing with neurones or the addition of nerve extracts to aneural cultures will alter the contribution from the major ions to overall resting membrane conductance.

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